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GRK2 AS A NEW ONCO-MODULATOR OF BREAST TUMOURAL TRANSFORMATION THROUGH THE REGULATION OF THE P53/MDM2 AXIS

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A mi hermano

"El azar afortunado suele ser casi siempre el premio del esfuerzo perseverante "

Santiago Ramón y Cajal

Los tónicos de la voluntad: Reglas y consejos sobre investigación científica. 1899

"That was the beginning, and the idea seemed so obvious to me and so elegant that I fell deeply in love with it. And, like falling in love with a woman, it is only possible if you do not know much about her, so you cannot see her faults. The faults will become apparent later, but after the love is strong enough to hold you to her. So, I was held to this theory, in spite of all difficulties, by my youthful enthusiasm."

Richard Feynman

Nobel Lecture, 1965

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ABSTRACT

The complex interactome of GRK2 points to this kinase as a key node in the signal transduction network of the cell, contributing to the proper functioning of basic cellular processes such as cellular migration, cell cycle progression or angiogenesis. Both GRK2 expression levels and activity are frequently found unbalanced in diverse diseases, thus suggesting a role for this kinase in aberrant cellular processes related to the onset or development of these pathologies. In the present work, we have extensively characterized the role of GRK2 as a novel onco-modulator of breast tumoural transformation, with particular focus on its impact in the Mdm2/p53 axis and /or in HDAC6-mediated responses. We have determined that high-amplification of ErbB receptors such as EGFR or Her2 and over-activation of the PI3K/AKT axis as a result of mutations in PI3K-p110, PTEN or AKT genes (all frequent molecular features of luminal types of breast tumours) converge in promoting GRK2 accumulation. Enhanced GRK2 levels are also triggered by the amplified oestrogen signalling characteristic of luminal tumours. Increased GRK2 expression, which is detected in transformed breast cell lines, in two murine mammary tumor models and in patients, would in turn activate two key regulatory axis /signalling modules with either Mdm2 or HDAC6 (also up-regulated in luminal breast cancer), leading to promotion of cell proliferation, survival and invasion in transformed cells. We find that ERK1/2-mediated modification of GRK2 at S670 enables GRK2-mediated HDAC6 phosphorylation and stimulation of its tubulin-deacetylase activity and microtubule dynamics, what results in higher persistence growth factor-dependent signalling and increased duration and extent of downstream stimulation of mitogenic and pro-survival molecules such as Ras, MAPK, AKT and Pin1. Moreover, our findings suggest that in breast tumoral contexts, GRK2 can act as a positive modulator of Mdm2 functionality. We describe herein that GRK2 specifically phosphorylates Mdm2 and potentiates the Mdm2-dependent wild-type p53 downmodulation, resulting in enhanced cell survival. On the other hand, the phosphorylation of GRK2 at S670 seems to cause a switch on the sub-cellular distribution of this kinase in tumour cells harboring mutant p53 (as luminal T47D, basal-A MDA-MB-468 or basal MDA-MB-231 cells), what could amplify oncogenic functions by means of the increased functional competence of Ras/Pin1 and HDAC6, which are involved in protecting mutant p53 from degradation and supporting its functionality. In fact, our data show that GRK2 function is required (in a p53 status-independent fashion) for enhanced proliferation, anchorage-independent growth, survival and invasion of tumoral breast cells "in vitro" and for tumor progression "in vivo", thus suggesting a relevant and general role for GRK2 as an onco-modulator of key processes involved in breast tumor development.

RESUMEN EN CASTELLANO

El complejo interactoma de GRK2 señala a esta quinasa como un nodo clave para la red de transducción de señales de la célula, participando en procesos básicos tales como la migración celular, la progresión del ciclo celular o la angiogénesis. Tanto los niveles de expresión de GRK2 como su actividad están sometidos a estrictos mecanismos de control que frecuentemente se ven alterados en diversos contextos fisiopatológicos, sugierendo un papel relevante de esta quinasa en la aparición o el desarrollo de estas patologías. En el presente trabajo se ha identificado a GRK2 como un nuevo onco-modulador de la transformación tumoral, responsable de estimular la adquisición de proliferación sostenida, crecimiento independiente de anclaje, supervivencia e invasividad de células de mama. Hemos caracterizado los mecanismos moleculares de la función onco-moduladora de GRK2, demostrando el papel crucial de esta quinasa en la regulación de la ligasa Mdm2 y la deacetilasa HDAC6 y sus consecuencias en la actividad de los módulos celulares de señalización de p53 y de Ras, cuya alteración es clave para la transformación celular. Nuestros resultados indican que la co-regulación al alza de GRK2/Mdm2/HDCA6 es una nueva característica molecular de los tumores de mama de tipo luminal que podría estar asociada a una mayor agresividad y peor prognosis. Hemos determinado que el exceso de actividad de la cascada de PI3K/Akt, como resultado de mutaciones en PI3K p110, PTEN o AKT o de la amplificación/sobre-estimulación de receptores ErbB, así como la sobre-activación de receptores de estrógenos, convergen en la acumulación de GRK2 en modelos celulares y animales y en pacientes. El aumento de la proteína GRK2 en líneas tumorales de mama luminales, junto con el de su fosforilación por ERK1/2 en el residuo S670 (característica también común a las líneas celulares derivadas de tumores basales) facilita y permite, respectivamente, la modificación por GRK2 de HDAC6, lo que conduce a la estimulación de la actividad tubulina deacetilasa y de la dinámica de microtúbulos, que a su vez lleva a potenciar la duración e intensidad de las señales mitogénicas y de supervivencia, así como a promover un fenotipo migratorio invasivo, estimulando la formación y funcionalidad de invadopodios. Por otro lado, nuestros resultados sugieren un nuevo mecanismo de regulación negativa del supresor tumoral p53 mediada por GRK2 a través del control directo de Mdm2. En contextos oncogénicos de mayor actividad Ras/ERK1/2 y/o PI3K/Akt, la interacción entre GRK2 y Mdm2 se reprograma con consecuencias funcionales distintas: estabilización de GRK2 y modulación de Mdm2 como nuevo sustrato de GRK2. Hemos identificado los sitios de fosforilación de Mdm2 por GRK2 y demostrado que estas modificaciones aumentan la estabilidad de la ligasa, promueven la interacción de la proteína con p53 causando la desestabilización de p53 y disminuyen las respuestas apoptóticas dependientes de este factor transcripcional. Además, la interrelación de GRK2 con Mdm2 y HDAC6 atenúa la capacidad citotóxica tumoral y anti-proliferativa de compuestos inhibidores de Mdm2 (Nutlin-3a) y de HDAC6 (tubacina, SAHA). Por último, la contribución de GRK2 a las respuestas mitogénicas y de supervivencia a través de la modulación de la actividad de Ras/Pin1 y de p53 vía Mdm2 y HDAC6 es necesaria para el crecimiento y progresión de tumores de mama "in vivo" tanto basales como luminales, sugiriendo un papel clave y global de GRK2 como onco-modulador de los diferentes procesos que dan lugar al desarrollo del cáncer de mama.

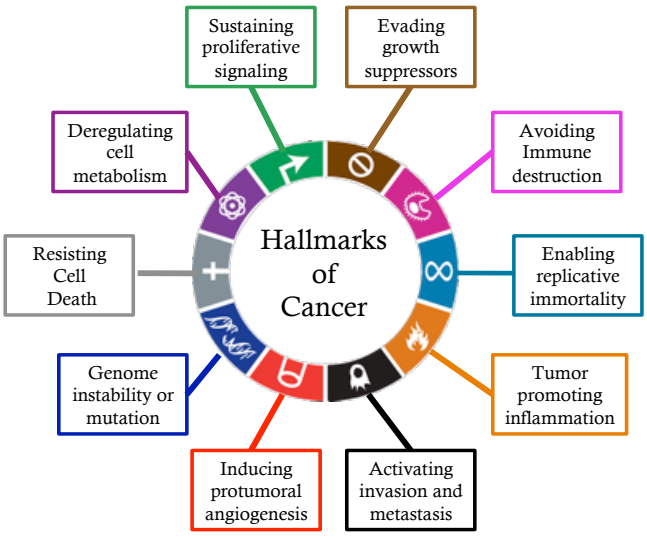
INTRODUCTION

1.

Hallmarks of cancer

Advances in Molecular Biology have revolutionized our understanding and knowledge of the pathogenesis of cancer, a disease that, until the 1990s, was mostly studied in purely descriptive terms. Nowadays, cancer is understood as a process caused by sequential genetic and epigenetic alterations that deregulate the activities of pathways mediating normal cellular functions, thus disrupting the regulatory circuits that govern normal cell proliferation and cell homeostasis. This multi-step process does not result from an abrupt transition from a normal to malignant condition, but instead involves progressive and gradual occurrence of molecular modifications and cellular adaptations by selective pressure over many years, what leads to considerable heterogeneity and variability among cancers. However, it has been proposed that most and perhaps all types of human tumours must acquire a succession of common capabilities that enable normal cells to become tumorigenic and ultimately malignant. These capabilities represent six essential alterations in cell physiology that collectively dictate malignant transformation and have been termed as "**hallmarks of cancer**" (Hanahan et al., 2000; Hanahan & Weinberg, 2011). These hallmarks were initially defined as: **maintained proliferative**

signalling, insensitivity to growth-inhibitory signals, resistance to cell death, limitless replicative potential, induction of angiogenesis and tissue invasion and metastasis. In the last decade two emerging hallmarks were added to this list: **metabolic reprogramming** and **evading immune response** (FIG. I.1). None of these hallmarks is sufficient by itself to promote transformation, but their intertwinement profoundly modifies the normal cellular behaviour and allows cells to survive, proliferate and disseminate. In addition, two transversal characteristics are permissive for the acquisition of these tumoural traits, thereby functioning as driving forces of cellular transformation. Such "tumour-enabling" properties include development of genomic instability and the chronic inflammation linked to pre-malignant and tumoural lesions. Impaired functionality of genome maintenance pathways underpins the increased mutation rate of cancer cells, what permits the rise of genetic changes that may orchestrate tumoural hallmarks. In turn, immune cells and inflammatory mediators can contribute to debilitate genomic surveillance mechanisms and to foster cancer progression by providing cell survival and proliferation support through direct impact on tumour cells or indirect remodelling of the surrounding stroma. Indeed, contributions of non-tumoural components (extracellular matrix, fibroblasts, resident immune cell types or vascular cells) are important for tumour growth, and feed-back reciprocal interactions between the neoplastic cells and the supporting stromal cells are at the root of high-grade malignancies. Therefore, tumour biology cannot be longer build on a simple recapitulation of capabilities but instead must consider contributions of the ''tumour microenvironment', what adds another dimension of complexity in the acquisition of cancer traits.



Adapted from Hanahan and Weinberg, 2011

Figure I.1. Hallmarks of cancer. Graphical representation showing the identified skills adquired by tumour cells that are necessary for cancer growth and progression.

2.

Physiology and complexity of breast cancer

Breast cancer is one of the most common cancers with more than 1,300,000 cases and 450,000 deaths each year world-wide and the most frequently diagnosed neoplastic process in women (Banin Hirata et al., 2014). Breast cancer is not a simple or uniform disease, but represents a heterogeneous disease including different histopathological entities with different molecular signatures, genetic and genomic variations (Ellsworth et al., 2009.).

Physiologically, the breast is an organ which structure reflects its special function: the production of milk for lactation. Mammary glands consist of 15–20 epithelial lobes, each of which develops numerous milk-producing lobules upon pregnancy (Fig I2A). Each lobule and lobe is connected to the nipple via ducts that transport milk. The lobules and ducts consist of a bi-layered epithelium comprising an inner layer of milk-producing luminal epithelial cells and an outer layer of myoepithelium that contracts to generate milk flow. Besides this function, breast myoepithelial cells play an important role in the suppression of tumour invasion as they form a natural fence separating the menstrual-cycling proliferative epithelial cells from the stroma. Nutrients are supplied to the breast tissue through a rich-

network of blood vessels, primarily derived from the internal mammary artery, while the interstitial protein-rich fluid is returned to the bloodstream by the lymphatic vessels of the breast that follow blood vessels in an opposite flow direction, to drain into lymph nodes.

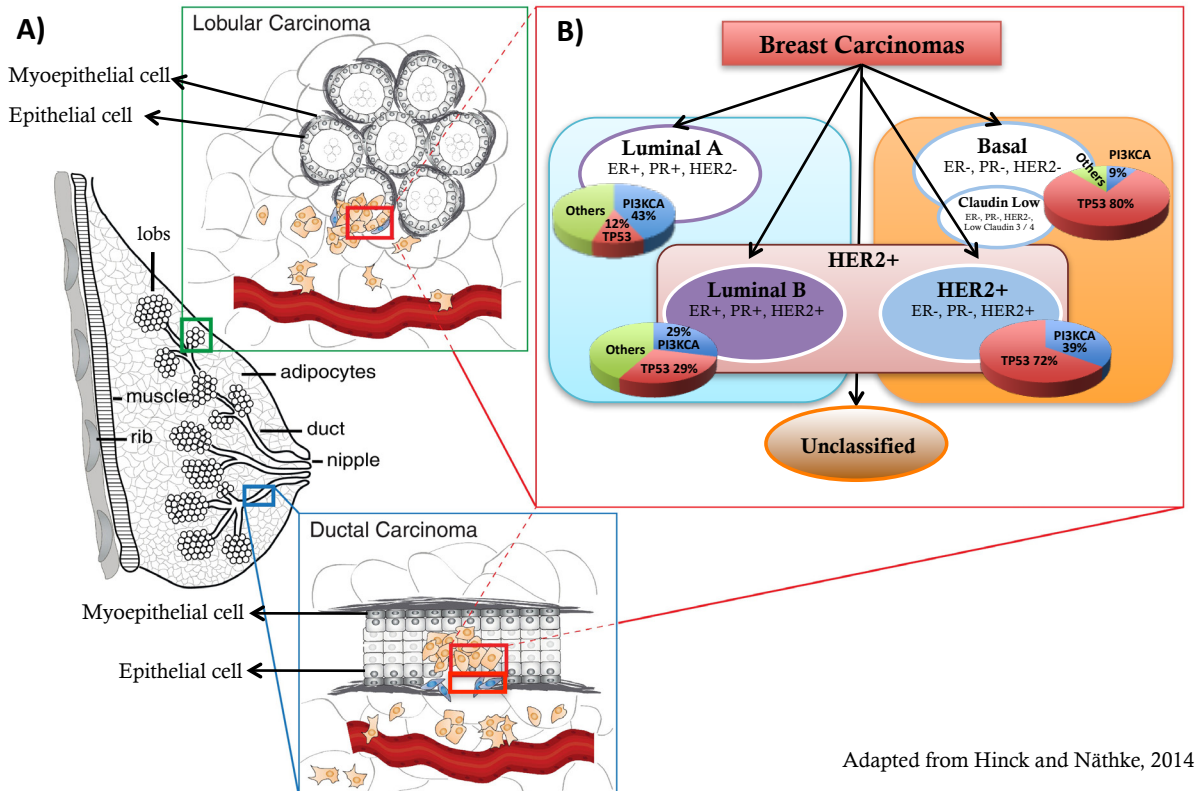


Figure I.2. Breast cancer classification. A) Schematized view of the tissue organization in mammary gland with an expansion of the cell types that develop breast cancer. B) Classification of breast cancer according to the molecular features of each tumour, showing the different proportions of mutations in key genes deregulated in the onset of the disease.

The first classification of breast cancer is based on the identity of the tissue component that gives raise to the tumour. According to that, carcinomas and sarcomas are the two main groups. Carcinomas arise from the epithelial component of the breast (i.e., cells that form the lobules and terminal ducts) and comprise the vast majority of all breast cancers, whereas sarcomas are less common cancers that originate from the stromal (connective tissue) component of the mammary gland. The most common type of breast carcinoma is the ductal carcinoma in situ (DCIS), a non-invasive lesion that contains abnormal cells inside of the pre-existing normal ducts, which precursor is thought to be the Atypical ductal hyperplasia (ADH), a pre-malignant lesion characterized by monotonous proliferation of regularly arranged cells within the duct or lobule. In those hyperplasias that are associated

with an increased incidence of malignancy, it appears that the luminal epithelial cells or the still-uncharacterized basal stem cells are the targets for transformation. Interestingly, myoepithelial cells have the property of self-renewal and it has been proposed that halt of stem cell differentiation into this cell type contributes to tumorigenesis (Pandey, 2011). In situ carcinoma has significant potential to become invasive and develop an infiltrating ductal carcinoma (IDC), with cancer cells infiltrating outside the normal breast ducts and growing into the breast connective tissue until they finally spread to other sites of the body. In such dissemination process the lymphatic drainages of the breast and the intercostal veins plus the vertebral plexus are of great importance to colonize in the form of metastasis lymph nodes and bones or the nervous system, respectively, among other organs (Vargo-Gogola & Rosen, 2007).

3.

Molecular classification of breast cancer.

Based on distinctive molecular profiling, breast ductal carcinomas, that comprise 80% of all breast cancers, can be grouped according to the Perou's classification in luminal A, luminal B, ERBB2+ (or HER2+) and basal-like, also referred as triple negative because they are negative for ER, PR and ERBB2 and associated with poor outcome and prognosis (Holliday & Speirs, 2011) (Fig 2.B). Luminal and basal subtypes display molecular similarities to the luminal and myoepithelial cells of the normal mammary gland, respectively. Thus, basal-type breast cancers are enriched in myoepithelial cytokeratins 5/6, 14 and 16, whereas luminal-type tumours harbour luminal markers (estrogen receptor-alpha, GATA-3 and cytokeratins 8/18), what would suggest that the former tumours arise from the underlying basal (myoepithelial) layer of normal breast ducts and the latter from the luminal secretory layer. However, this simplistic notion has been called into question since the previously accepted basal-layer origin for Brca1 mutant breast cancer has turned into ER-negative luminal origin (Molyneux et al., 2010), suggesting that luminal progenitor cells might trans-differentiate into basal cells or de-differentiate to a bipotent progenitor/ stem cell.

Irrespective of the histological origin of the transformed cell, both A and B luminal types of breast cancer are positive for expression of ER, but luminal B is enriched in ERBB2 and p53 mutations, thereby exhibiting increased proliferation and worse prognosis. In contrast, 80% of basal-like tumours present mutations in TP53 (mostly non-sense mutations). Together with the RAS/MAPK pathway triggered by high-level amplification/upregulation of growth factor receptors (ERBB2, IGF1R, EGFR), the PI3K pathway is also a major contributor to the uncoupled cellular proliferation and increased survival of breast tumour cells (Banerji et al., 2012; Cancer genome atlas network, 2012). A significant proportion of human breast tumours of luminal origin displays increased activity of the PI3K/AKT signalling axis by means of mutations in PIK3CA and AKT genes, while the remaining basal-like display only 9% PI3KCa mutations. However, genomic loss of PTEN is a common feature for basal-like tumours. The ERBB2 subtype presents Her2/ErbB family amplification and frequent p53 and PI3KCa mutations or loss of PTEN (Banerji et al., 2012; Cancer genome atlas network, 2012). Moreover, CyclinD1 amplification occurs with high incidence in luminal tumours, mostly in luminal B subtypes (Holliday & Speirs, 2011), whereas a novel subtype was recently described based on the low levels of claudin (Herschkowitz et al., 2007). This last group displays all the features of the basal tumours but differs from the latter in the distinctive downregulation of claudin-3 and 4, low expression of the KI67 proliferation marker and enrichment for markers associated with the epithelial–mesenchymal transition and with mammary cancer stem cells, making these tumours more aggressive and highly metastatic (Holliday & Speirs, 2011; Kao et al., 2009). Breast tumour cell lines often mirrors the heterogeneity of the different types of tumours from which they derive, but basal cells lines can be additionally stratified in two subgroups, A and B as well (Kao et al., 2009). Interestingly, although tumours of the basal A subtype are triple-negative (ER-, PR- and ERBB2-), they share some luminal epithelial markers, what leads to a tumoural behaviour closest to that of luminal groups than to the more related basal B cell lines.

From a clinical point of view, breast tumour oestrogen receptor-positive patients are the more "treatable" group, since they can respond to endocrine therapies based on antagonists of the ER pathway. On the other hand, the HER2/ERBB2-amplified group of patients also can undergo successful clinical treatments with anti-ErbB2 therapy, whereas triple negative breast cancers lack specific molecular-targeted treatments and patients can only receive classical chemotherapy (Kao et al., 2009). However, some tumours can develop resistance to known therapies or, as mentioned before, relative resistance appears from cross talk between signalling pathways. Thus, a deep characterization of the molecular features and behaviour of the tumours is essential to find a specific directed therapy against each tumour.

4.

Most relevant oncogenic pathways in breast cancer

To better understand breast cancer, a detailed study about the molecular changes that are taking place in cells as they become malignant is absolutely required. As mentioned before, there are several hallmarks common to cancer cells, but the underlying molecular mechanisms and signalling pathways engaged to promote these tumoural capabilities vary from one tumour to another. On the basis of gene expression signatures several modules within the cellular signalling network can be identified as core oncogenic signalling pathways and as those carrying frequently mutated genes. Such modules include the Ras, p53 and TGFβ signalling blocks with their related transducing hubs and regulatory loops (Cui et al., 2007). These oncogenic blocks control key tumour-related functions such as cell cycle control, cell proliferation and apoptosis. In this section, we summarize the most relevant signalling pathways related to these blocks in breast cancer, with a particular focus on their contribution to the acquisition of cancer hallmarks. It is worth noting that the functionality and competence of these blocks is also determined by the extent of post-translational modifications, including phosphorylation, acetylation, ubiquitination or SUMOylation of regulatory and signaling hubs. In this context, the goal

of this thesis project is to gain insight into the functional interplay of the serine-threonine kinase GRK2 and the ubiquitin-ligase Mdm2 and the relevance of such interplay in breast tumour biology.

4.1. Oncogenic pathways controlling sustained proliferative signalling and cell survival

It is worth noting that the most fundamental feature of cancer cells is their ability to sustain chronic proliferation. Normal tissues finely control the production and release of growth-promoting signals that direct entry into and progression through the cell cycle, thereby ensuring cell number homeostasis (Hanahan & Weinberg, 2011). However, cancer cells deregulate these signals through the over-expression or the acquisition of activating mutations on several proteins (encoded in so-called oncogenes), what leads to uncontrolled cell growth.

Oncogene-driven proliferation of tumoural cells is assisted by various mechanisms. First, cells can produce their own growth factors or stimulate the continuous release of such factors by neighbouring normal cells within the tumour-associated stroma (Cheng et al., 2008; Bhowmick et al., 2004) . Alternatively, growth factor-receptor proteins displayed at the cancer cell surface can also be mutated or over-expressed, rendering such cells hyper-responsive to otherwise-limiting amounts of growth factor ligands. Constitutive activation of signalling components operating downstream to these receptors may drive cellular growth factor-independent proliferation, thus overcoming the need of ligand-mediated receptor activation (Hanahan & Weinberg, 2011). Consequently, growth factor receptors, protein kinases regulators, GTP-protein signalling switchers, and transcription factors of specific signal transduction pathways for which particular cells display mitogenic-addiction are common targets for oncogenic transformation.

a. Growth factor receptor signalling: tyrosine kinase receptors as a common platform for multiple oncogenic pathways

Cell surface receptors that transduce growth-stimulatory signals into the cell are themselves targets of deregulation during tumour pathogenesis. Growth Factor (GF) receptors, often harbouring tyrosine kinase activities in their cytoplasmic domains, are hyper-activated in approximately 20–30% of human breast cancers (Iglehart et al., 1990). In particular, gene-amplification or over-expression of members of the human ErbB receptor family (constituted by four members: ErbB1 (EGFR/ HER1), the orphan receptor ErbB2 (HER2 or Neu), ErbB3 (HER3), and ErbB4 (HER4)) and the increased expression of activating growth factors such as EGF (ErbB1 ligand) or heregulin/neuregulin (ErbB3/

ErbB4 ligands) are commonly observed and their occurrence has been closely associated to the incidence and poor prognosis of breast cancer (Citri, 2003; Hynes & Lane, 2005).

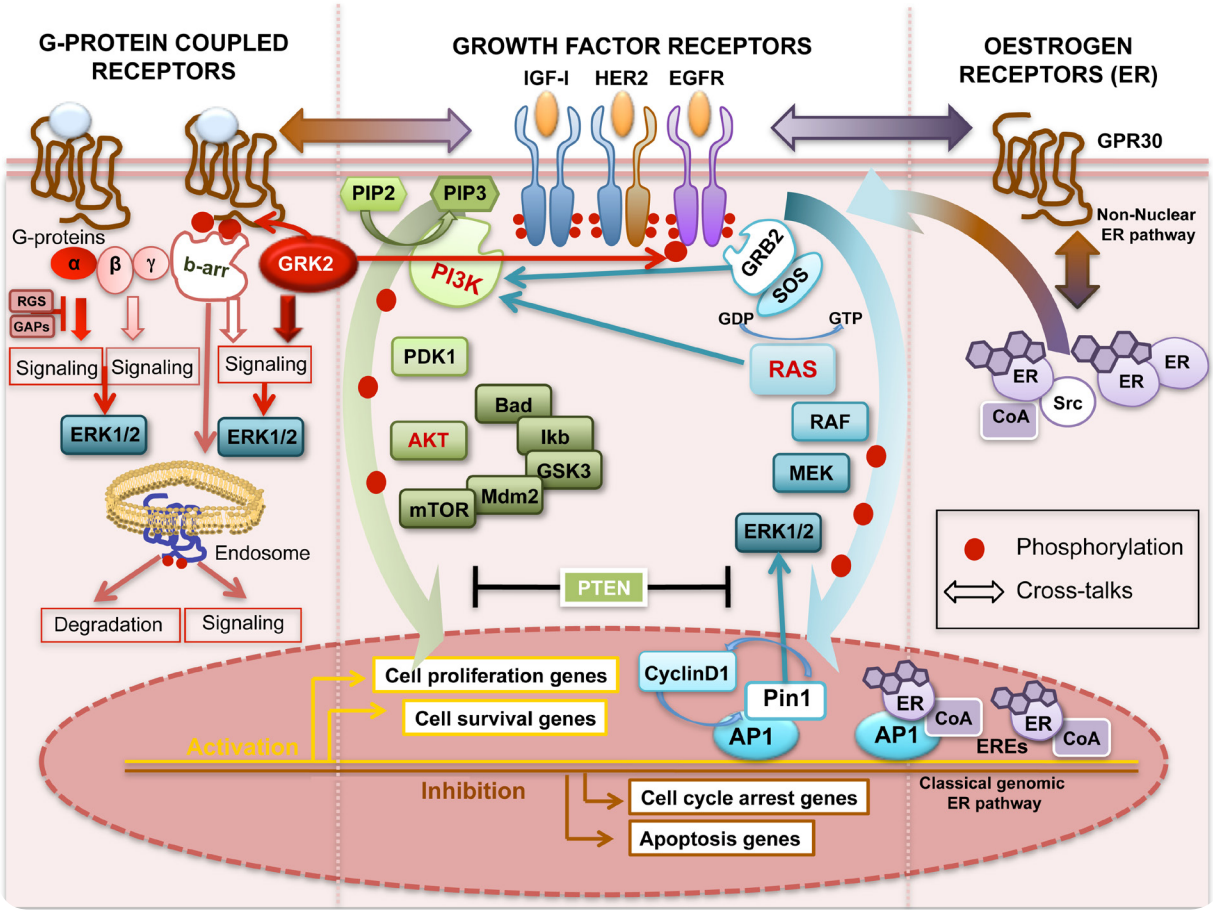


Figure I.3. Oncogenic signalling governing breast tumour transformation. Growth factors receptors, G-protein coupled receptors and Oestrogen receptors are the main mediators of the growth and survival signals that trigger oncogenic transformation. In this scheme, the main down-stream signalling pathways and the inter-relations between them are represented.

Upon agonist binding to their extracellular domains, ErbB receptors can homo- or hetero-dimerize. Their combinatorial dimerization and subsequent phosphorylation together with their distinct coupling to signalling adaptors (through SH2 domains) and effectors create a complex network of signalling events (Sosa et al., 2010). Regarding the ErbB2 receptor, no physiological selective ligands have been identified, but it is activated by cognate ligands of the other members of the EGFR receptor family by means of their heterodimerization (Olayioye et al., 2000). ErbB receptors can signal through AKT, MAPK, and many other pathways to regulate cell proliferation, differentiation, apoptosis, and cell motility (Fig. I.3).

b. Signal transduction mediated by the ErbB receptor family: Mutations and / or alterations of the Ras-Raf-MEK-ERK and PI3K / AKT signalling modules

It is well established that members of the family of small GTP-binding proteins such as Ras mediate most of the ErbB responses. Upon ligand-induced trans-phosphorylation, the ErbB receptor associates with the growth factor receptor bound 2 (GBR2) adaptor, which triggers the recruitment of the protein SOS. Subsequently, SOS acts as a GEF (guanine nucleotide exchange factor) and catalyzes the exchange of GDP for GTP in the nucleotide-binding pocket of RAS-GTPases, thus resulting in their activation. In the activated GTP-bound state, Ras is able to bind to and activate several effectors proteins, such as the kinase b-Raf and the p110 catalytic subunit of PI3K (Rodriguez-Viciana et al., 1994; Steelman et al., 2011) (Fig. I.3). Genetic abnormalities associated with breast cancer include gain-of-function mutations of ErbB effectors. Moreover, PI3KCA gene mutations or PTEN deletions are common features in many cases of breast cancer (Banerji et al., 2012). Most notably, many Ras responses depend on cross-talking connections between both RAS/MAPK and PI3K pathways, increasing the likelihood and robustness of cell proliferation and survival (Cully et al., 2006; DeNicola & Tuveson, 2009; Eckert et al., 2004; Margolis & Skolnik, 1994). Ras mutations are rarely seen in breast cancers (5%) but, still, there is considerable experimental evidence that aberrant Ras activation and signalling can promote breast cancer development (Eckert et al., 2004). Thus, in breast cancers Ras may be activated by persistent upstream signalling instead of through direct mutational activation. Defective EGFR downregulation and intracellular misrouting can drive such signalling persistence. Indeed, balance between recycling and degradation is determined by a regulatory cluster of Ser and Thr residues in the EGFR that is modified by several kinases including ERK1/2 and p38 or by the less characterized GRK2 (Freedman et al., 2002; Zhang et al., 2011). Thus, post-translational modifications including phosphorylation or ubiquitination and protein-protein interactions directly modulate EGFR functionality and can contribute to neoplastic cell transformation.

i. The ErbB-dependent mitogenic signalling

Downstream ErbB receptors, active b-Raf phosphorylates and stimulates mitogen-activated kinases (MEK) 1 and 2, which in turn phosphorylate the kinases ERK1 and 2 (Margolis & Skolnik, 1994) (Fig I.3). Finally, the kinase cascade leads to the activation of transcription factors of the AP1 family among others, promoting the expression of many genes that encode growth factors, cyclins and cytokines which, collectively, trigger cellular proliferation (Mechta et al., 1997). One of the most important final effectors of the Ras/Raf/ERK pathway is Cyclin D1, which is in fact amplified in most human breast cancers, playing a pivotal role in the development of the disease by promoting cell cycle progression

(Caldon et al., 2010; Lee et al., 2000; Liu et al., 1995; Zeng et al., 2010). Additionally, it has been recently shown that the prolyl-isomerase Pin1, also frequently up-regulated in breast cancer, is another downstream target of the oncogenic Neu/Ras signalling and essential for Neu/Ras-induced cyclin D1 activation and cell transformation. Indeed, the upregulation of Pin1 in the context of Neu/Ras signalling was reported to enhance β-catenin and c-Jun signalling, leading to transactivation of the cyclin D1 gene. Furthermore, Pin1 is also able to directly bind cyclin D1 and to stabilize it via post-transcriptional mechanisms (Ryo, 2003; Ryo et al., 2002; Wulf et al., 2001) (Fig. I.3). It is possible that cyclin D1 also regulates Pin1 expression via E2F in a positive feedback loop (Ryo et al., 2002). Moreover, Pin1 can also amplify EGF signalling in breast cancer cells through its interaction with MEK1 thereby enhancing HER-2 expression, in another positive feedback loop (Khanal et al., 2010). Finally, it is well known that Pin1 acts on various cell cycle proteins to coordinate progression through the cell cycle (Wulf et al., 2001). Thus, Pin1 operates as a novel molecular key of the Neu-Raf-Ras-MAPK signal pathway by acting on multiple targets at various steps of the signal network to promote cell proliferation and transformation.

ii. PI3K/AKT pathway

The PI3K/AKT pathway is a highly intertwined network that arises as an integrator of multiple inputs derived from RTKs and other membrane receptor types during breast tumorigenesis (Cully et al., 2006). Besides the Ras-dependent activation of PI3K that might be common to different receptor systems, tyrosine-phosphorylated RTKs act as docking platforms for direct or Grb2-Gab1-mediated indirect engagement of the regulatory p85 subunit of PI3K and subsequent kinase activation (Fig.I3). Active PI3K catalyses the transformation of membrane-bound phosphatidylinositol (4,5) bisphosphate (PIP2) into the second messenger phophatidylinositol-(3,4,5) tris-phosphate (PIP3). Since PIP3 is restricted to the plasma membrane, this results in the translocation of the serine-threonine kinase AKT (also called PKB) to the plasma membrane, where it is activated by phosphorylation in Thr308 by PDK1 and in Ser473 by PDK1, mTOR or AKT itself (Cidado & Park, 2012; Steelman et al., 2011) (Fig.I.3). Recently, a novel mechanism accounting for full AKT activation through S477/T479 modification by mTOR and CDK2/cyclinA has been described in response to DNA damage that allows cell cycle progression and chemoresistance (Liu et al., 2014). AKT is a proto-oncoprotein with multiple effectors (Bad, Ikb, GSK3, mTOR....) that control crucial biological processes such as apoptosis, protein synthesis or translation, proliferation and cell survival (Kim & Chung, 2002). Therefore, the activity of AKT is tightly regulated in physiological conditions. The multifunctional phosphatase PTEN acts as a negative regulator of the pathway through the transformation of PIP3 to PIP2, blocking the activation of AKT. PTEN can also interact with and dephosphorylate

ERK1/2, thereby inhibiting cell proliferation (Cully et al., 2006). AKT-1 kinase activity contributes to the development of breast cancer and to therapeutic responses as tumour cells with acquired resistance to anti-tumour agents may display higher AKT activation and treatments based on molecular-directed targeting can often activate feed-back loops involving AKT (Cancer genome atlas network, 2012).

c. G-Protein coupled receptors(GPCRs) as drivers of breast cancer proliferation and growth: RTK-transactivation- dependent and independent mechanisms.

G protein-coupled receptors (GPCRs), also known as seven-transmembrane domain receptors or 7TM receptors, constitute the other major protein family of receptors which deregulated activity have been linked to aberrant proliferation and metastatic progression of tumours of different nature (Dorsam & Gutkind, 2007; Lappano & Maggiolini, 2011). The first evidence of the potential oncogenic roles of GPCRs came from the identification of activating oncogenic mutations in the thyroid stimulating hormone receptor (TSHR) and from the connexion of virally-encoded GPCRs with tumorigenesis. In addition, potent mitogens such as thrombin or PGE2 regulate tumour cell proliferation and/or invasion through binding to their cognate GPCRs (PAR1 and EP2/EP4) and the stimulation of Rho/Rac modules and of MAPK cascades including ERK, JNK, p38 and ERK5 (O'Hayre et al., 2014). Interestingly, these receptors are usually over-expressed or their regulatory processes altered in breast tumours (Boire et al., 2005; Chang et al., 2005; Filardo et al., 2008). GPCRs lack intrinsic catalytic activities but couple to heterotrimeric G proteins, molecular transducers composed of βγ dimers and α subunits of four subfamilies, each of which directly modulates a specific set of enzymes (adenylase cyclases activated or inhibited by Gαs or Gαi, phospholipases activated by Gαq and small GTPases activated by G12/13, among others) to generate second messengers and stimulate diverse signalling cascades. Both G protein-dependent signalling and receptor/G protein coupling ability are tightly controlled by the heterotrimeric G protein cycle and the receptor homologous desensitization process, respectively (Fig. I3). Ligand-bound GPCRs act as GEFs for Gα subunits, facilitating the GDP/GTP exchange process. GTP-bound α subunits dissociate from the βγ dimer, and both G protein subunits regulate an ever-expanding list of independent effectors. Finally, GTP hydrolysis, a process known to be increased by RGS (Regulator of G-protein signalling) and GAP (GTPase-activating proteins) proteins, causes re-association of G protein subunits and termination of signal transduction. Besides this regulatory G protein cycle, receptor signalling is also curtailed by the ligand-induced engagement of the desensitizing factors GRKs and arrestins. Phosphorylation of agonist-bound GPCRs by GRKs leads to the translocation and high affinity binding of the cytosolic

proteins β-arrestins to the receptors (Benovic et al., 1986; Premont et al. 1995). This event not only inhibits further G protein-receptor interactions, but also allows the recruitment of the endocytic machinery, ultimately leading to receptor internalization, which can promote either GPCR-resensitization or receptor degradation.

Besides the stimulation of mitogenic pathways by G-protein and second messengers-dependent mechanisms (for instance activation of ERK1/2 by cAMP- and PKA-stimulated Rap1 or by Calcium-stimulated calmodulin kinase), several other signaling mechanisms appear to contribute to GPCR-mediated MAPK activation (Fig.I.3). These include transactivation of the EGFR via either the autocrine/paracrine release of EGF-like ligands at the cell surface or the direct Src-mediated activation of the receptor (independently of EGF ligand shedding). For instance, EGFR are transactivated by oestrogen via the G-protein-coupled receptor GPR30 (Filardo et al., 2008; Prossnitz et al., 2007), defining a novel signalling pathway with potential significance for proliferation of (ERα)-negative human breast cancer cells. Another mechanism involves G protein-independent, β-arrestin-mediated activation of MAPK cascades, what means that beyond its classical desensitization function, β-arrestins have the ability to scaffold diverse signaling complexes including the Raf-MEK-ERK1/2 module in a c-Src-mediated manner and the ASK1/MKK4/JNK3 module. Activation of β–arrestin signalling is biased by particular GPCR ligands and linked to specific patterns of GRK-mediated receptor phosphorylation that trigger different receptor conformations for β-arrestin docking. ERK activated via β-arrestins differs from the G protein-activated one in several aspects as mitogenic potency, intracellular location or signalling time-course. Therefore, it is not surprising that changes in the expression and localisation of arrestins (and of GRKs, see section 6) may occur during cancer progression. In this regard, high levels of both β-arrestins have been described in advanced-stage breast cancer, correlating with reduced patient survival (Sobolesky & Moussa, 2013). Another layer of complexity derives from the fact that RTKs and GPCRs can form platforms in which protein signalling components specific for each receptor are shared to produce an integrated response upon engagement of ligands (Pyne & Pyne, 2011). For instance, PDFGβR might use G proteins to transmit signals to effectors upon formation of membrane complexes with the GPCR S1P1. On the other hand, heregulin binding to ErbB2 activates a β-arrestin-biased CXCR4 signalling, independently of its cognate CXCL12 ligand, through a direct tyrosine receptor trans-phosphorylation (Sosa et al., 2010), while CXCR4 can associate with other RTK, such as IGF-RI, in response to IGF, leading to preferential coupling of CXCR4 to Gαi proteins (Akekawatchai et al., 2005). Overall, combined receptor platforms and reciprocal hijacking of signal transducers have the potential to reprogram intracellular signalling and to alter the outcome of conventional pharmacological receptor antagonists in the context of cancer.

d. Oestrogen receptors: the endocrine contribution

Oestrogen-induced proliferation of normal cells has been considered a milestone for the initiation and promotion of tumorigenesis (Blanco-Aparicio et al., 2007). Cellular actions of oestrogenic compounds are mediated by two "classical" nuclear estrogen receptors α(ERα) and β (ERβ), which expression is present in 75% of breast cancers (Fig.I.3). Nuclear ERs act as transcriptional factors regulating the expression of genes required for cell division and survival (Frasor et al., 2004). Upon oestrogen binding, ER homo or hetero-dimerizes and attracts a complex of co-activators and co-repressors to specific sites on DNA (oestrogen response elements, EREs) (Klinge, 2001). ER can also bind to other transcription factors such as AP-1 and SP-1 at their specific sites on DNA, thereby functioning as a co-regulator (Babu et al., 2013; Bartella et al., 2012). However, a non-genomic ER activity has been suggested, since the rapid oestrogen-dependent activation of PI3K/AKT and Ras/ERK1-2 pathways (Osborne et al., 2013) is not compatible with the slower time-course of ER-triggered transcriptional changes (Soltysik & Czekaj, 2013). These non-genomic effects are mediated by plasma membrane-associated estrogen receptors (mER), and the cellular transmembrane G protein-coupled estrogen receptor (GPER/GPR30). Although mERs do not have intrinsic kinase activity, they can orchestrate a membrane "signalosome" (Moriarty et al., 2006), where numerous molecules potentially important for mediating rapid signalling cascades, such as G-proteins, tyrosine kinase c-Src, modulator of non-genomic activity of the ER (MNAR), caveolin-1, and heat shock protein 90 (Hsp90) interact in response to estrogens. In this context a robust crosstalk between mER and co-recruited growth factor receptors has been described. Moreover, oestrogen receptor can increase the expression of ligands such as transforming growth factor-α (TGFα) and IGF1 or directly increase IGF1-R levels (Hawsawi et al., 2013; Vyhlidal et al., 2000) which can then activate the growth factor receptor pathway (Giuliano et al., 2013; Nicholson et al., 2005; Osborne et al., 2013) In the absence of ER expression, oestrogens can activate GPR30 and stimulate MAPK and PI3K/AKT signaling through either G protein and adenylyl cyclase-mediated or EFGR transactivation-dependent mechanisms (Filardo et al., 2008; Prossnitz et al., 2007).

4.2) Oncogenes and neutralization of tumour suppressors by loss-of-function mutations/loss-of-expression provides cells with death resistance features: the p53 / Mdm2 axis

Cancer cells are insensitive to anti-growth signals, displaying unlimited replication, and usually harbour inactivating mutations in tumour suppressor genes whose products normally contribute to DNA repair processes. Indeed, the most common cause of hereditary

breast cancer is related to mutations in one of such genes, Brca1. In addition, tumour suppressors may be also involved in the detection of genomic and metabolic stresses or in the activation of cell-cycle checkpoints and proliferation arrest. Their inactivation involves considerable vulnerability, since cumulative replication cycles increase the frequency of single- or double-strand DNA breaks and chemical modifications (intrinsic replicative stress) on cells that have deficient mechanisms to cope with them. However, in contrast to normal cells which undergo apoptosis when are unable to repair DNA insults and damage become excessive for genomic integrity, transformed cells disconnect the cellular death response by inactivating additional tumour suppressors that either act as pro-apoptotic regulators (Chk2) or negative regulators of survival pathways (PTEN by blocking the PI3K/AKT pathway). Consequently, cells continue to grow and divide as they accumulate mutations, thus contributing to the process of tumoural transformation (Alexander & Friedl, 2012).

The most widely impaired tumour suppressor in human cancer is p53, a potent transcription factor also known as "the guardian of genome" that is activated in response to diverse types of stress and environmental insults, leading to the induction of cell-cycle arrest, apoptosis or senescence (Fuster et al., 2007; Levine & Oren, 2009; Vogelstein et al., 2000) . The p53 protein possesses an acidic N-terminal transactivation domain with an intervening hydrophobic conserved box I (aa 13-18), a proline-rich domain, and a centrally located sequence-specific DNA-binding domain bearing conserved boxes II-V, followed by an oligomerization domain and a basic C-terminal regulatory domain (Fig.I.4). Wild-type p53 functions as a homo-tetramer in cells, binding to p53 response elements in a myriad of genes. Among the regulatory mechanisms controlling p53, the proteasome-dependent degradation appears to be the most relevant, since the knock-out mice for Mdm2, the main E3 ligase responsible for p53 ubiquitination and degradation through the proteasome pathway (Finlay, 1993; Vogelstein & Kinzler, 1992), is embryonic lethal due to an exacerbated expression of p53 (de Rozieres et al., 2000). However, many other ubiquitin ligases including Pirh2, COP1, CHIP and ARF-BP1 have been identified as regulators of p53 in response to different signalling pathways, thus underscoring the importance of keeping at bay p53 levels in unstressed conditions (reviewed at Carter and Vousden, 2009). In contrast, upon cellular stresses p53 undergoes phosphorylation, acetylation and other types of modifications that prevent ligase action. Such modifications also affect protein activity and subcellular localization, leading to p53 up-regulation and activation (Bode & Dong, 2004). The extent and repertoire of such posttranslational modifications is proportionate to the severity and nature of the stress and serves to direct p53's actions toward particular sets of gene responders and protein partners. p53 activated by milder DNA-damages transactivates components of the mismatch and the nucleotide excision repair

system and of cell cycle arrest like p21Cip1 or GADD45 (Menendez et al., 2009), whereas stronger DNA damage or persistent lesions trigger a p53-mediated programmed cell death by inducing the transcription of pro-apoptotic proteins such as Noxa, Bax and PUMA among others (reviewed in (Nag et al., 2013; Riley et al., 2008; Vousden & Lu, 2002)). p53 is also involved in the ATM-mediated induction of senescence (Armata et al., 2007; Efeyan et al., 2009). In addition, p53 can also act as a transcriptional repressor of oncogenes that promote cell growth, survival or angiogenesis, such as c-fos, myc or VEGF-A (Nag et al., 2013) . Recently, new functions of p53 have been discovered including p53 roles in energy metabolism (Maddocks & Vousden, 2011), immune response, cell differentiation, motility and migration and cell-cell communication, that, together with the well known functions in apoptosis, cell cycle regulation, senescence and angiogenesis makes this transcription factor a master regulator of the cell biology (Fuster et al., 2007; Muller et al., 2011; Riley et al., 2008). Thus, it is not surprising that in over 50% of all human cancers TP53 gene is mutated or deleted, while in the remaining tumours the wild-type p53 function is inhibited by several other mechanisms, as DNA tumour viruses encoding proteins that can inactivate p53, or up-regulation/activation of negative modulators such as Mdm2 leading to p53 downmodulation (Cancer genome atlas network, 2012).

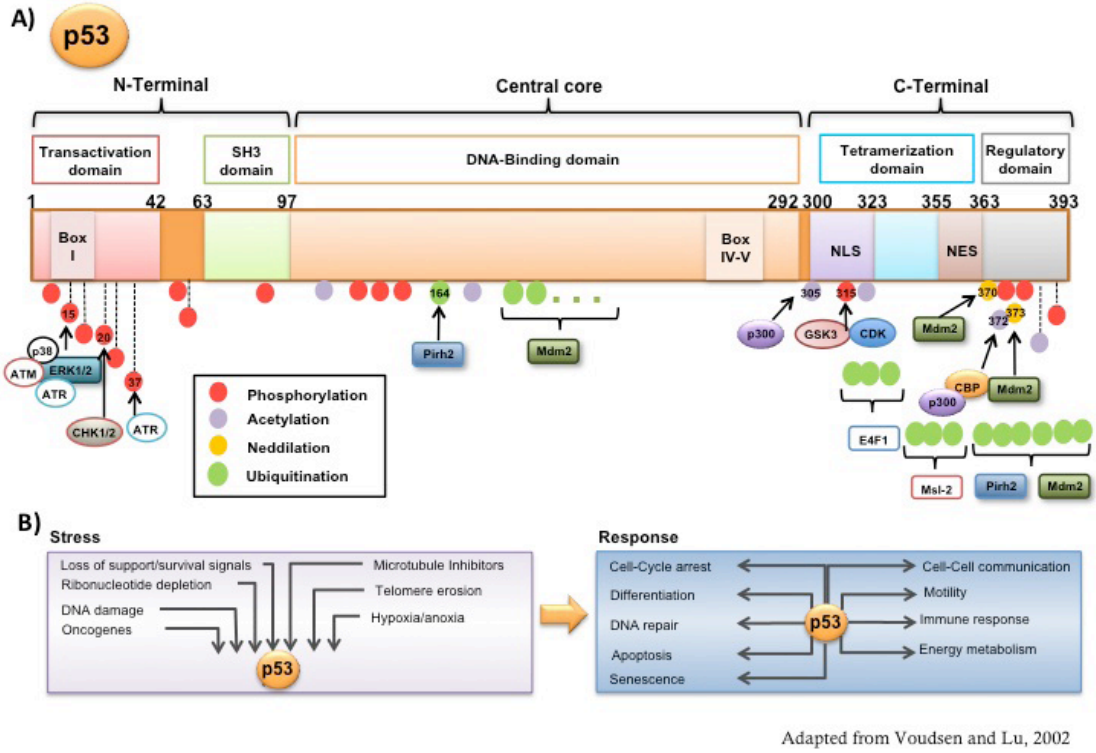


Figure I.4. p53 structure and post-transcriptional modifications. A) Linear representation of p53 sequence with relevant domains and the main post-transcriptional modifications. B) Several stresses lead to p53 activation, which can result in different cellular responses.

a. The functional interplay between Mdm2 and p53

Mdm2 is a multidomain protein endowed with multiple functional activities that go beyond the mere ubiquitination of p53. The most N-terminal part of Mdm2 harbours the lid domain (aa 1-24), a flexible region that folds over and regulates in a pseudosubstrate-like fashion the hydrophobic pocket (aa 25–108), which binds p53, among other partners. The central region of Mdm2 includes nuclear localisation (NLS; aa 179–185) and nuclear export signals (NES; aa 190–202), followed by an acidic domain (CAD, aa 243–301) that partially overlaps with the zinc finger domain (aa 290–335). In the very C-terminus is located the RING (for "really interesting new gene") domain (aa 432–491) that promotes its E3 ubiquitin ligase activity, harbours its nucleotide binding site and also encompasses the nucleolar localisation signal (Fig.I.5).

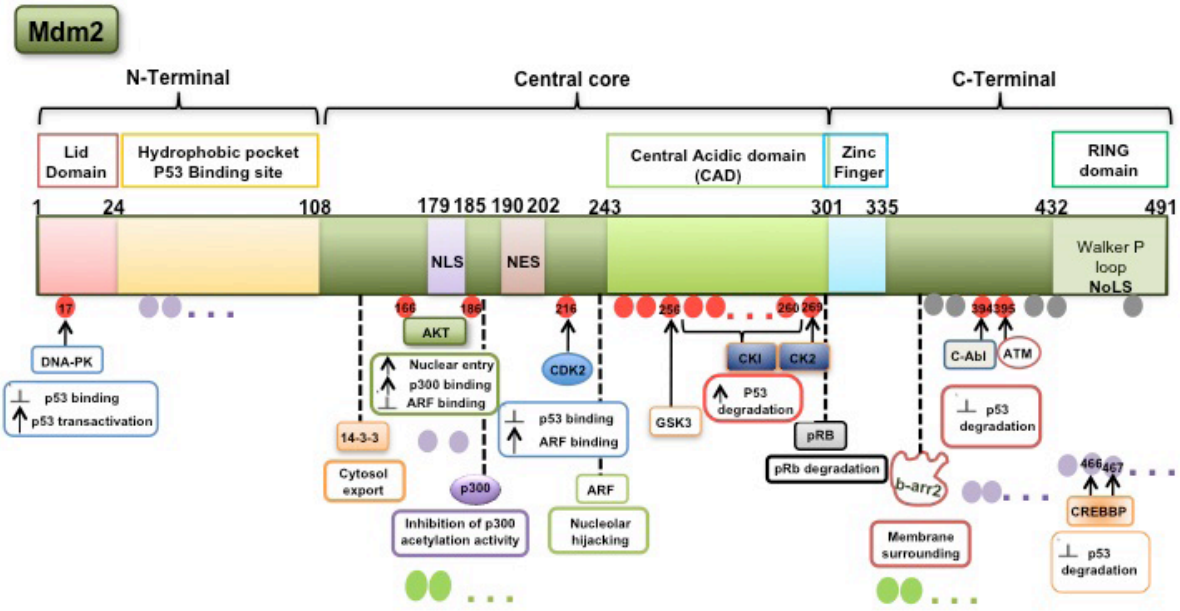


Figure I.5. Mdm2 structure and post-transcriptional regulation. Linear representation of Mdm2 sequence with relevant domains and the main post-transcriptional modifications, showing the functional consequences of these alterations.

Mdm2 and p53 form a unique negative-feedback loop, in which p53 induces the expression of Mdm2 by binding to its promoter (Vousden & Prives, 2009), while Mdm2 quenches cellular p53 activity by altering its stability, localization and transactivation activity (Nag et al., 2013) (Fig.I6). Thus, the mutual binding of Mdm2 and p53 via the N-terminal hydrophobic domain and the box1 region, respectively, directly blocks the p53 transactivation domain. This first interaction is followed by subsequent contacts involving the acidic domain of Mdm2 and the Box V region of p53, which produce allosteric conformational changes that allow the RING domain of Mdm2 to either mono-ubiquitinate or poly-ubiquitinate p53. In fact, the interaction between the N-terminal domains of Mdm2 and p53 is not required for the Mdm2-catalyzed p53 ubiquitination, but instead the second interaction is critical for such modification (Ma et al., 2006). Mono-ubiquitination of several Lys residues in the p53 C-terminus acts as a signal for p53 nuclear export to the cytosol, wherein it displays scaffolding regulatory functions on apoptotic proteins (Fig. I.4 and I.6). On the other hand, poly-ubiquitinated p53 is quickly degraded by the proteasome pathway (Lai et al., 2001). Potential ubiquitin acceptor sites in p53 also includes other regions of the protein and poly-ubiquitination within the DNA-binding domain seems to be responsible for protein destabilization (Chan et al., 2006). It is reported that the Mdm2 capability of catalyzing both mono-ubiquitination and poly-ubiquitination of p53 is dependent on the levels of Mdm2, with poly-ubiquitination occurring at high doses of Mdm2 (Lai et al., 2001). By its own, Mdm2 is a poor ligase for p53 poly-ubiquitination, thus requiring the assistance of additional factors to form ternary complexes in which the RING domain of Mdm2 is competent to elicit such modification on p53. Among these factors, MdmX (or Mdm4), a paralog of Mdm2, is an essential, non-redundant, p53 regulatory protein. Although the RING domain of MdmX is unable to ubiquitinate p53, it is involved in the activation of the Mdm2-dependent poly-ubiquitination of p53 via hetero-dimerization with the RING domain of Mdm2 (Wade et al., 2010) (Fig. I.5). Other factors engaged in ternary activating complexes related to p53 regulation are the proteasome activator PA28y protein, which increases Mdm2-p53 interaction (Zhang & Zhang, 2008) or UBE4B, an E4 factor of the E3 U-box family that binds to Mdm2 and p53 and facilitate poly-ubiquitination. Recently, the p53-acetylating protein p300 was reported to be also an E4 ligase that mediates by itself poly-ubiquitination on previously mono-ubiquitinated and cytosol-exported p53 (Shia et al., 2009) .

Beyond the well-known role of Mdm2 in the ubiquitination and degradation of p53 through the proteasome pathway, other levels of p53 regulation have been reported, as binding of Mdm2 impedes p53 interaction with transcriptional co-activators or recruits transcriptional co-repressors to p53. It has also been described a chaperone-like function

of ATP-loaded Mdm2 that assists p53 assembly on the p21 promoter (Wawrzynow et al., 2007). Moreover, the RING domain bears a conserved Walker A or P-loop motif characteristic of nucleotide binding proteins that allows binding of Mdm2 to the nuclear p53 mRNA under genotoxic stress. This prevents MDM2 from targeting p53 for degradation while increases the rate of p53 mRNA translation.

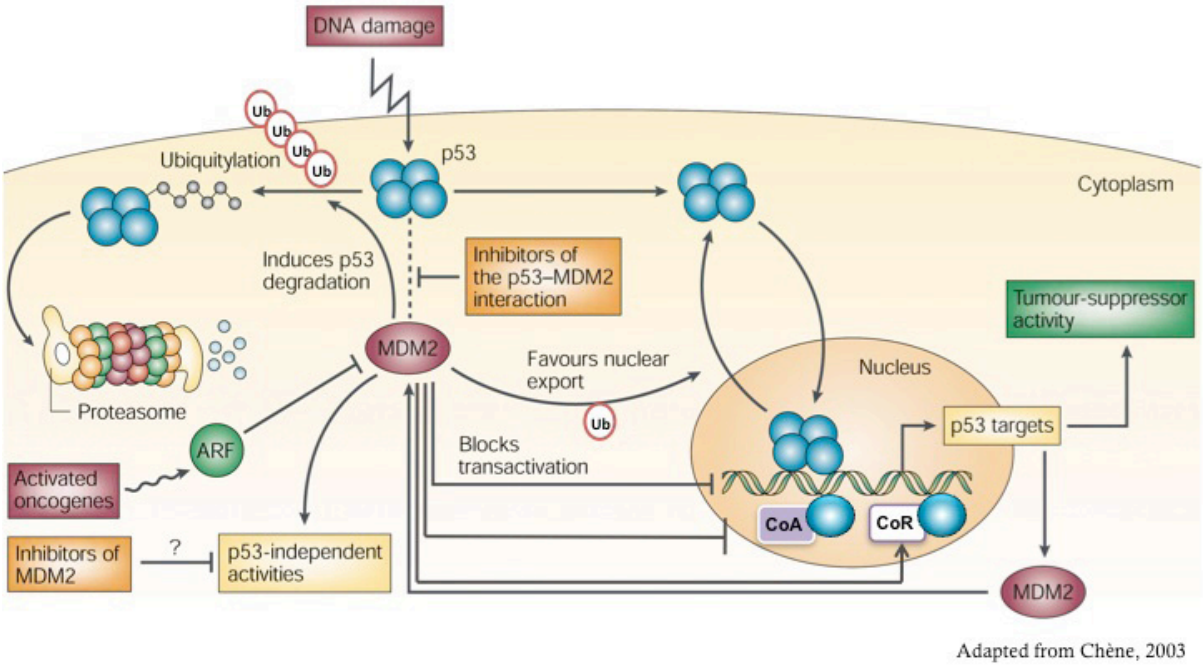


Figure I.6. Mdm2/p53 axis. p53 and MDM2 form an auto-regulatory feedback loop. Different cellular stresses such as DNA damage induce p53 activation by preventing its MDM2-mediated degradation. Consequently, p53 stimulates the expression of p53 targets and iniciates its tumour suppressor activities. Interestingly, and as a regulatory mechanism, it also promotes the transcriptional expression of Mdm2, which in turn inhibits p53 activity, by mono- or poly-ubiquitinating p53 and favouring its nuclear export and its degradation, respectively. Moreover, Mdm2 is also able to block p53 transcriptional activity.

b. Regulation of the ability of Mdm2 to target p53

Regulation of p53 by Mdm2 is a crucial step in cell homeostasis and, consequently, Mdm2 is a target of a wide variety of tumour suppressors and oncoproteins and modifying enzymes. Mdm2 displays multiple post-transcriptional modifications that affect protein functionality (Coutts et al., 2009). First of all, Mdm2 promotes its own ubiquitination and subsequent degradation when it homodimerizes in the absence of MdmX (Lai et al., 2001), whereas sumoylation has the opposite effect on stability and regulates ligase nuclear and nucleolar localization. Neddilation has been described as a novel modification in Mdm2 that blocks the function of Mdm2 as transcriptional suppressor of p53, resulting in increased expression of p53 (Xirodimas et al., 2004). In addition, acetylation of lysine clusters within

the MDM2 RING domain has been shown to attenuate the E3 ligase activity of MDM2 towards p53 (Fig.I5).

Extremely important is the modification of Mdm2 by different kinases, what may "reprogram" the functionality of Mdm2 in particular cellular settings. Most of the phosphorylation sites of Mdm2 are clustered in the central acidic domain. Importantly, some phosphorylation events in this region are related to allosteric conformational changes in Mdm2 required for an efficient p53 binding to the N-terminal domain of the ligase and for full ubiquitination activity towards p53 (Meek & Knippschild, 2003). However, phosphorylation sites lie also on other unstructured and inter-domain regions of the ligase, such as between the Zinc and RING finger or around NLS and NES localization signals, which modification can impose conformational changes to modify/stabilize interacting interfaces. Thus, DNA damage-response kinases (ATM, ATR) or the kinases responsible for the detection of replicative failure (Chk2 and c-Abl) phosphorylate Mdm2 near the RING domain, inhibiting the oligomerization of Mdm2 important for its E3 ligase activity and unmasking RNA-binding sites in the ligase, what overall contributes to p53 accumulation (Chehab et al., 2000; Maya et al., 2001; Shinozaki et al., 2003). On the other hand, IGF1R or HER2-induced AKT-mediated phosphorylation of Mdm2 in serines 166 and 186 promotes Mdm2 nuclear translocation (Jackson et al., 2006) and increases the ligase function against p53 (Ogawara et al., 2002).

Finally, subcellular distribution of Mdm2 is strongly regulated through interactions with several proteins such as p14ARF or PML among others (Bernardi et al., 2004; Pan et al., 2011; Reed et al., 2014; Xirodimas et al., 2004). The ARF-Mdm2 interaction sequesters Mdm2 in the nucleolus, while 14-3-3σ causes translocation of the ligase to the cytosol (Bernardi et al., 2004; Lee & Lozano, 2006). Moreover, Mdm2 association with oligomers of β-arrestin2 promotes Mdm2 trafficking from nucleus to cytosol, and upon ligand challenge Mdm2 is brought via β-arrestins into the vicinity of activated GPCRs and RTKs such as IGF1R (Boularan et al., 2007; Wang et al., 2003), allowing the access of the ligase to other substrates. Furthermore, the serine 166 has a role in controlling the p53 and ARRB1 ternary complex that is induced via the β2-adrenoreceptors to suppress DNA damage-induced stress response (Hara et al., 2011). On the other hand, a subset of Ribosomal proteins (RPLs) bind to the CAD and Zn-finger domains of Mdm2 to inhibit its ligase activity toward p53 as part of the nucleolar response pathway activated by metabolic stress (Deisenroth & Zhang, 2011).

c. P53-independent functions of Mdm2

It is well established that over-expression of Mdm2 provides cells with a growth advantage, promotes tumorigenesis, and correlates with worse clinical prognosis and poor response to cancer therapy (Manfredi, 2010). In fact, Mdm2 over-expression or its functional amplification is a frequent event in neoplastic diseases (5% of all human tumours and more elevated percentage in the case of breast cancer) (Efeyan et al., 2007). Moreover, the higher incidence of breast tumours in p53 knockout mice that are also transgenic for Mdm2, and the occurrence of high levels of Mdm2 in the presence of mutant p53 or even in the absence of p53 gene expression in patients with worse prognostic suggests additional roles of Mdm2 in the oncogenic transformation that are independent of p53 regulation (Manfredi, 2010).

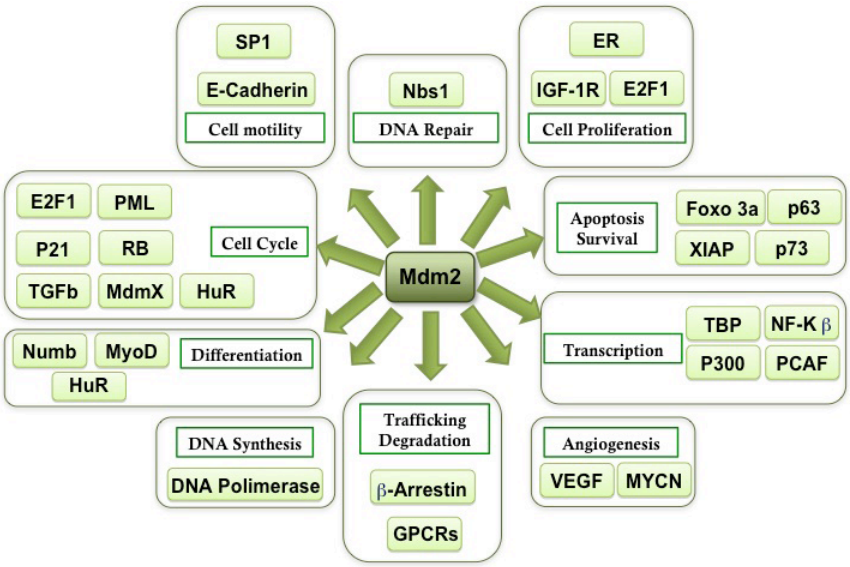


Figure I.7. p53-independent functions of Mdm2. Besides its function as a negative modulator of p53 response, Mdm2 also participate in a wide variety of biological processes through the ubiquitination of other sustrates.

Mdm2 can interact with at least 70 different protein partners by means of its different domains, and regulate the functionality of many of those, even in an ubiquitination- or catalytic-independent way (Shi & Gu, 2012) (Fig.I.7). The functional MDM2 interactome involves factors that divert its E3 ligase activity towards other substrates but in a way still affecting p53 activity, such as MDMX, also a substrate of Mdm2. Other interactions can affect MDM2 E3 ligase activity unrelated to p53 modulation, whether others may alter Mdm2 functions not depending on E3 ligase activities. Indeed, Mdm2 is able to catalyze other modifications such as protein neddylation on itself and on other substrates such as p53 or HuR (Embade et al., 2012). This versatility might be related to the fact

that the Mdm2 RING domain is atypical when compared to canonical RING ligase domains. Its conformational plasticity allows this region to couple to E2 activities for ubiquitin and Nedd8, to engage mRNA sequences leading to stabilization of VEGF or MYCN transcripts and bind nucleotides such as ATP in order to behave as a molecular chaperone of transcriptional complexes of ER or E2F1 factors with certain promoters (Slack et al., 2005; Stevens et al., 2008; Wawrzynow et al., 2007; Xirodimas et al., 2004; Zhou et al., 2011), Moreover, the diverse conformations that Mdm2 can adopt induced by different combinations of protein modifications or regulatory interactions, the different subcellular localizations and the occurrence of splice variants and hetero-oligomerization can contribute to govern the specificity of Mdm2 partner interactions and their functional outcomes.

The complex interactome of Mmd2 underpins the influence of this protein in several signalling pathways and different biological responses (Fig.I.7). Mdm2 might participate in the cell transformation by regulating nuclear proteins such as p73, p63, p21, pRb, p300, E2F, PCAF (Gu et al., 2001; Jin et al., 2003, 2004; Miwa et al., 2006; Uchida et al., 2005; Zeng et al., 2003) or membrane proteins such as the IGF-1R receptor (Girnita et al., 2003; Yin et al., 2011) or E-cadherin (Yang et al., 2006). Recently, novel Mdm2 substrates have been described, what increases the possibility of finding novel p53-independent functions for Mdm2. For example, ERK dependent-phosphorylation of Foxo3a turns Foxo3a into a target for Mdm2-dependent ubiquitination and degradation. As Foxo3A regulates expression of genes that encode cell cycle regulators such as p27, this leads to a Mdm2-mediated control of cell cycle progression in response to oncogenic growth factor signalling or Ras activation in a p53-independent way (Yang et al., 2008). Mdm2 has also been shown to regulate the expression of the anti-apoptotic protein XIAP by a novel mechanism, which involves the binding of Mdm2 to the mRNA that encodes XIAP, thus enhancing its translation (Gu et al., 2009). Interestingly, oestrogens are found to be transcriptional effectors of Mdm2 up-regulation in breast cancer cells (Brekman et al., 2011; Okoro et al., 2013). Interestingly, oestrogen can also activate cell proliferation using Mdm2 to repress multiple cell cycle checkpoints without the involvement of p53 (Brekman et al., 2011). Overall, available data indicate that Mdm2 can regulate key aspects of cell proliferation and apoptosis in both a p53-dependent and independent manner.

d. TP53 mutations: Gain of oncogenic functions

TP53 is the second most frequently mutated gene after the PI3KCA proto-oncogene in mammary tumours, becoming a key-driving factor in triple negative breast cancer. Most of the alterations in TP53 are missense mutations that are overrepresented in the central

DNA-binding domain, affecting in spontaneous tumours mainly to codons R175, C220, G245, R248, R273 and R280 (Fig.I.8). Mutation of p53 not only hampers the tumour suppressor functions of this factor, but also leads to the acquisition of oncogenic properties by gain-of-function mechanisms. While some hotspot mutations impede contact between p53 and DNA, others distort the DNA-binding domain resulting in an altered promoter activation spectrum that involves both abnormal transactivation of particular p53 wt-dependent genes (such as p21 or Mdm2) that are useful for tumour cell maintenance, as well as targeting of additional genes lacking p53-reponse elements that favour tumour progression by increasing invasiveness and chemoresistance. Mutant p53 can also directly activate transcription of specific micro–RNAs and attenuate micro-RNA processing (Suzuki et al., 2009). Moreover, mutant p53 cooperation with different transcription factors such as NFkB, NF-Y or E2F1 may be an important route to execute its gain-of-function activity. Finally, although wt p53 does not hetero-oligomerize with its paralogs p73 and p63, mutant p53 can via its DNA-binding domain, what presumably attenuates the anti-tumorigenic functions of these related factors, further contributing to cell transformation.

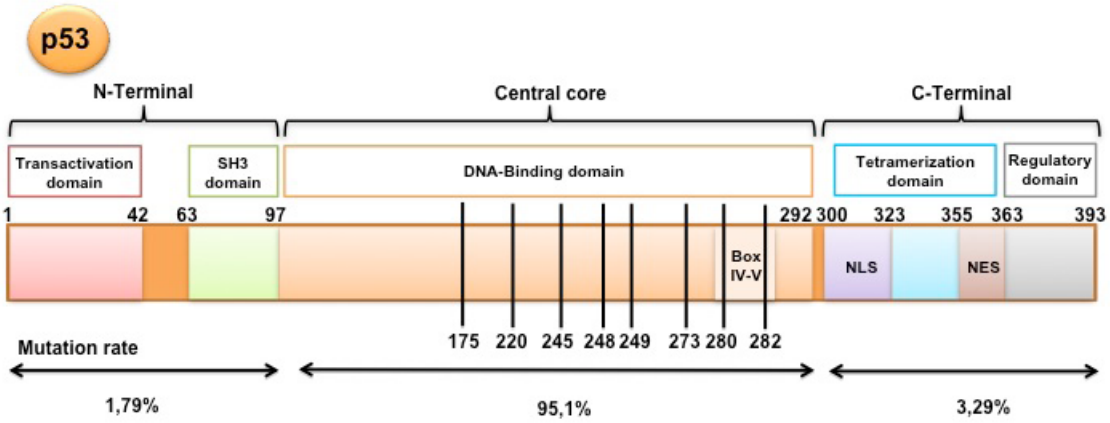


Figure I.8. Location of tumour-associated p53 mutations. Almost all of the point mutations that are found in cancers occur within the central DNA-binding core of p53; the percentage of mutations within each region detected in cancers to date is indicated below and the most relevant mutated residues are highlighted.

Signalling pathways controlling mutant p53 levels are not entirely understood. The activity of mutant p53 is regulated through upstream signal transducers as well as regulators of transcription, stability and structure. While ER-alpha was shown to inhibit wt p53-dependent transcription, hormone-triggered complexes involving mutant p53 may potentially influence the transcriptional spectrum of cells expressing mutant p53. On the other hand, the prolyl-isomerase Pin1, a component of checkpoints mechanisms

that increases wt p53 stability upon DNA damage (Ryo, 2003) also plays a role in the stabilization and gain of function of mutant p53 by means of the enhancement of p53 pro-migration and invasion activities, in a mechanism that involves the p53 paralog p63 (Girardini et al., 2011; Muller et al., 2011; Wulf et al., 2002).

e. Mdm2 and mutant p53 protein stability: Oncogen or tumour suppressor?

Oncogenic and genotoxic stresses can modify mutant p53 on the same regulatory sites as on wt p53, leading to similar stabilization mechanisms. However, even though mutant p53 can be efficiently ubiquitinated and targeted for degradation by MDM2 as well as by other E3 ligases, several mechanisms seem to counteract theses processes in tumoural contexts. The interaction between the chaperone Hsp90 and mutant p53 stabilizes protein conformations and blocks its degradation by inhibiting Mdm2 activity (Li et al., 2011a). Moreover, p16INK4 downmodulation, a common event in tumour progression, also cause mutant p53 stabilization (Terzian et al., 2008). In this context, opposite to its function as oncogene, growing evidence argues for the remarkable possibility that Mdm2 has tumour suppressor functions by means of either the promotion of mutant p53 degradation (Prives & White, 2008; Suh et al., 2011) or the nuclear export of wild-type p53, which facilitates p53 binding to members of the Bcl2-family in the mitochondria, activation of mitochondria-dependent apoptosis and cell death (Vaseva et al., 2009; Vaseva & Moll, 2009).

5.

Cell Invasion and Metastasis:

From cell migration to invadopodia formation

Tumour cell invasion into host tissues and local and metastatic dissemination underlay the poor prognosis of certain breast cancers. Cellular invasiveness is dependent on the capacity of cancer cells to migrate from the tumour mass, to disrupt the basement membrane and remodel the extracellular matrix (ECM), followed by intravasation into blood or lymphatic vessels and subsequent metastasis to distant organs (Fig.I.9). Many cell types are able to undergo molecular and morphological polarization and to trigger motion in response to chemotactic gradients. Such oriented migration or chemotaxis is fundamental for embryogenesis, immunity and wound healing (Petrie et al., 2009). However, it also contributes to pathological conditions such as cancer or inflammatory diseases (Müller et al., 2001). Directed cell migration is dependent on a dynamic sequence of cell-substrate attachment at the leading edge of the cell coordinated with cell-substrate detachment at the rear (Frame et al., 2002). For a cell to migrate, it must initially sense and respond to a stimulus by developing filopodia and lamellipodia structures at the leading edge. Cell lamellipodium builds strong adhesion points with the substrate

and protrudes its membrane forward by polymerizing actin in the direction of movement. The formation of focal adhesions close to the leading edge of the cell provides the traction required to move the cell body forward. At the back, the actomyosin cytoskeleton contracts the cell body and breaks the adhesion points. Forward cell movement is then accompanied by focal-adhesion disassembly and cell-surface detachment at the rear of the cell, which permits retraction of the tail during active migration.

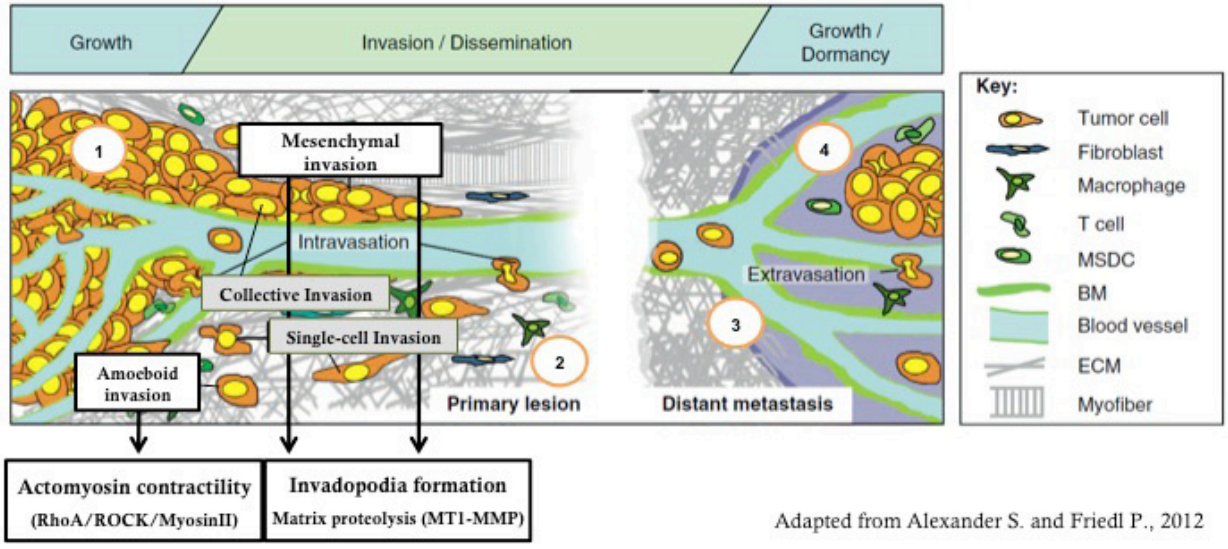


Figure I.9. The metastatic cascade. Mechanisms of tumor invasion and metastatic dissemination. After tumour formation (1) at the primary site, single cell or collective invasion lead to the breakdown of the basement membrane and intravasation into local vessels. Tumoural cells can also go through the membrane using a contraction- based amoeboid motility (2). Whereas the mesenchymal invasion requires the formation of protusive structures called invadopodia and MT1-MMP-dependent matrix proteolysis, amoeboid invasion relies in RhoA/ROCK/MyosinII-mediated actomyosin contractility. Before arrest in secondary organs, tumor cells extravasate (3) and regrow to macroscopic metastases (4). In both primary tumor and metastasis, reactive stromal cells such as fibroblasts, macrophages, myeloid- derived suppressor cells and regulatory T cells (Treg) contribute to cell growth, survival and invasion by the release of soluble factors and extracellular matrix remodeling. Abbreviations: BM, basement membrane; ECM, extracellular matrix; MDSC, myeloid-derived suppressor cell.

Cancer cells may adopt different ways of invasion depending on the cell type, the environment and the intrinsic molecular signature (Poincloux et al., 2009). The first detectable evidence of invasion is the degradation of the basal membrane, a thin layer that separates the epithelium from the stroma and that is basically formed by Collagen Type IV. This can be produced by the formation of protrusive structures called invadopodia. Invadopodia formation requires dynamic remodelling of f-actin and cortactin to transport metalloproteinases (mostly MT1-MMP) to the cell surface, where they are able to degrade the matrix. Invadopodia are generated by formation of dendritic actin networks but elongate as a result of extension of bundled longitudinal actin fibers. This elongation is

accompanied by contact with microtubules and vimentin intermediate filaments, both of which support the maturation of the structure (Linder et al., 2011).

Pericellular proteolysis mediated by metalloproteinases, particularly by MT1-MMP, allows tumour cells to remodel the matrix, supporting invasive migration through the 3D fibrillar collagen network (Poincloux et al., 2009). Proteolytic tracks left behind by invasive cells might support the migration of other cells. A second strategy used by cancer cells to invade relies on collective cell migration, in which cells migrate as a multicellular sheet, strands, files or clusters, maintaining cell-cell contacts. Recently, a novel invasion mechanism was characterized based on an amoeboid motion (Poincloux et al., 2011). This kind of invasion does not depend on metalloproteinase activity and matrix proteolysis, but rather it is driven by RhoA/ROCK and MyosinII-dependent contractility, allowing cells to acquire a rounded morphology and to squeeze between gaps in the 3D matrix (Fig.I.9). Epithelial tumours commonly use collective migration mechanisms, although both single cell invasion and collective moves are found simultaneously in many tumours, depending on their differentiation stage (the more un-differentiated, the less collective), their molecular signature or the environmental cues, in response to which cells might adopt different invasiveness strategies. This "plasticity" allows cells to shift from highly adhesive to low adhesive migration, from proteolytic or mesenchymal to non-proteolytic (amoeboid), from collective to individual migration and vice versa (Friedl & Wolf, 2003).

Interestingly, signalling pathways controlling tumour cell growth and survival such as p53, RasGTPases, small Rho GTPases, integrins, growth factor receptors and cadherins (some of them briefly summarized in the sections above) also regulate the cellular components involved in migration and invasion, and their activation state influences the invasive plasticity of cells (Alexander & Friedl, 2012). Chemokine receptors of the GPCR family such as CXCR4 or CXCR2 (also called CCR7) play a central role in the migration and metastasis of breast cancer cells (Cabioglu et al., 2005, 2009; Ruffini et al., 2007). Moreover, Mdm2-dependent E-cadherin degradation (Yang et al., 2006) or of the action of Mdm2 as an enhancer of MMP9 expression can lead to increased extracellular matrix breakdown and tumour invasiveness (Chen et al., 2013b). Given the overlap between migration-inducing and pro-survival pathways, recent evidences point at an interconnection between cancer invasion and therapy resistance. Thus, acquiring a migratory state probably contributes to resistance induction and vice versa (Alexander & Friedl, 2012).

6.

Regulatory molecular nodes as ancillary players to support malignant transformation and breast cancer progression.

In addition to the oncogenic drivers described above, other cancer-associated factors that are not necessarily genetically altered can cooperate with oncogenic-signalling routes or act in normal signalling compensatory pathways to strength tumoural properties or to cope with intrinsic tumour-derived vulnerabilities. Regulatory molecular nodes can integrate multiple upstream inputs and elicit diverse downstream outputs, what makes them suitable as non-oncogenic contributors to malignant transformation and progression. In this context, the serine/threonine kinase GRK2 is emerging as a key node in oncogenic signal transduction networks. In agreement with this notion, GRK2 levels or functionality are found altered in granulose cell tumours, thyroid and prostate cancer (King et al., 2003; Métayé et al., 2002; Prowatke et al., 2007) and recently associated with the aberrant activation of the PI3K/AKT pathway in some mammary cell lines (Salcedo et al., 2006).

6.1. GRK2 as a key node in the modulation of cellular signalling networks

a.G-protein-coupled receptor kinases (GRKs): Family, structure and canonical function

G-protein-coupled receptor kinases (GRKs) are a family of serine-threonine kinases composed of seven members (GRK1 to GRK7) that share a global homology of 60-70% and are grouped in three subfamilies: visual GRKs, present in cones and rods, include GRK1 (or rhodopsin kinase) and GRK7; the β–Adrenergic receptor kinase family (β–ARK), to which GRK2 and GRK3 belong, and a third subfamily, which includes GRK4, 5 and 6 (Premont & Gainetdinov, 2007) (Fig.I.10). Non-visual kinases are ubiquitous, excepting GRK4, which is predominantly expressed in testes and, to a lower extent, in brain and kidney.

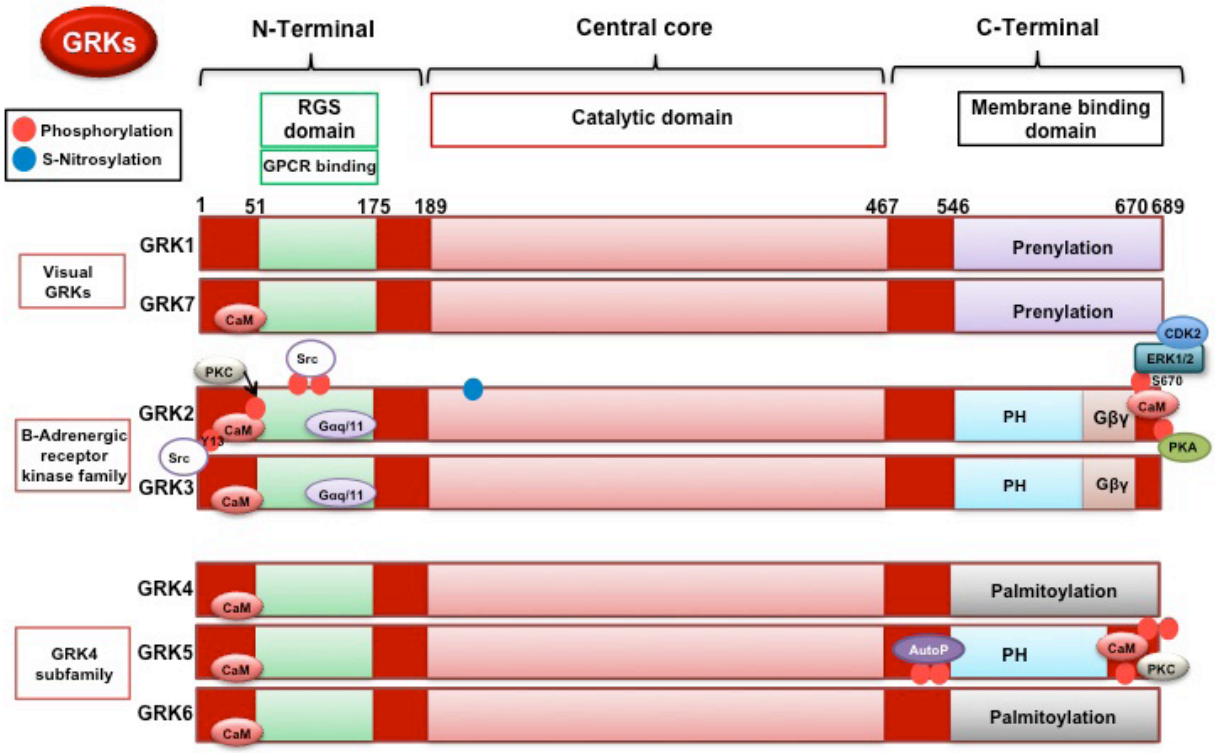


Figure I.10. GRKs family. Main domains of the three subfamilies of GRKs and localization of modulatory phosphorylation sites and regions involved in interaction with other proteins or the membrane are indicated.

GRKs have a multidomain structure, with a preserved central catalytic domain (so called KD for Kinase Domain) of around 500-520aa. The N-terminal domain spans 183-188 residues and, except in GRK1 and GRK7, includes a N-terminal RGS homology (RH) domain that , in the case of GRK2 and GRK3, has been shown to allow the interaction with Gαq/11 proteins (Carman et al., 1999). The GRK's C-terminal domain, more variable in extension and function, determines the localization of these proteins at the

plasma membrane. A pleckstrin homology domain (PH) in GRK2/3 proteins mediates their agonist-induced translocation from the cytosol to the plasma membrane through interactions with phospholipids and with G protein βγ (Gβγ) subunits at the membrane surface (Koch et al., 1993; DebBurman et al., 1996; Pitcher et al.,1992). On the contrary, GRK5 is predominantly associated to cellular membranes by means of basic sequences at the N-and C-terminus of the protein. GRK1/4/6 and GRK7 are constitutively anchored to the plasma membrane through short C-terminal prenylation sequences (GRK1 and 7) or through palmitoylation sites (GRK4 and GRK6) (Gurevich et al., 2012; Pitcher et al., 1996; Ptasienski, 1996). Interestingly, and unlike many other kinases, activation of GRKs does not require a previous phosphorylation in their activation loop. Instead, docking to active GPCRs and allosteric interactions with lipids directly triggers their kinase activity (Gurevich et al., 2012) (Fig.I.10).

As we have mentioned before, GRKs are involved not only in GPCR desensitization but also in the GPCR-mediated G protein-independent signalling through the engagement of arrestins to the receptor complex, which in turn recruits diverse components of signalling cascades. The mechanism by which GRKs determine whether to promote GPCR desensitization or G protein-independent signalling remains unclear, but it has been suggested that selective activation of specific GRKs or differential phosphorylation by particular GRKs of ligand-bound GPCRs (also termed "phosphorylation barcoding") might be implicated in recruiting arrestins with a conformation competent to scaffold specific signalling molecules. For instance, phosphorylation of the angiotensin receptor by GRK2 and GRK3 modulates beta-arrestin-mediated GPCR internalization, whereas phosphorylation by GRK5 or GRK6 triggers the beta-arrestin-dependent signalling to MAPK (Kim et al., 2005). However, GRKs are emerging as new signal transducers by themselves, performing β-arrestin-independent roles in GPCR signalling cascades and also arising as new players in non-GPCRs-dependent pathways, as a result of functional or scaffolding interactions with a wide variety of substrates (Penela et al., 2003; Ribas et al., 2007) (Fig. I.11).

b. The complex interactome of GRK2: GRK2 contributes to pathways controlling the hallmarks of cancer

GRK2 is a ubiquitous, essential, and best- characterized member of the GRK family. Recent evidences point at this kinase as a key node in the control of the most relevant signalling networks required for the proper function, homeostasis and viability of the cell. The fact that global GRK2 knockout mice are embryonically lethal (Jaber et al., 1996) further supports the notion that this protein plays a central role in such key cellular

processes. Non-GPCR proteins that interact and/or are phosphorylated with GRK2 include single transmembrane RTKs and a variety of cytosolic or nuclear signalling proteins, and the impact of this protein on cell functions can be mediated by either its catalytic activity or in a kinase-independent manner by acting as a scaffold (Penela et al., 2010a) (Fig.I.11).

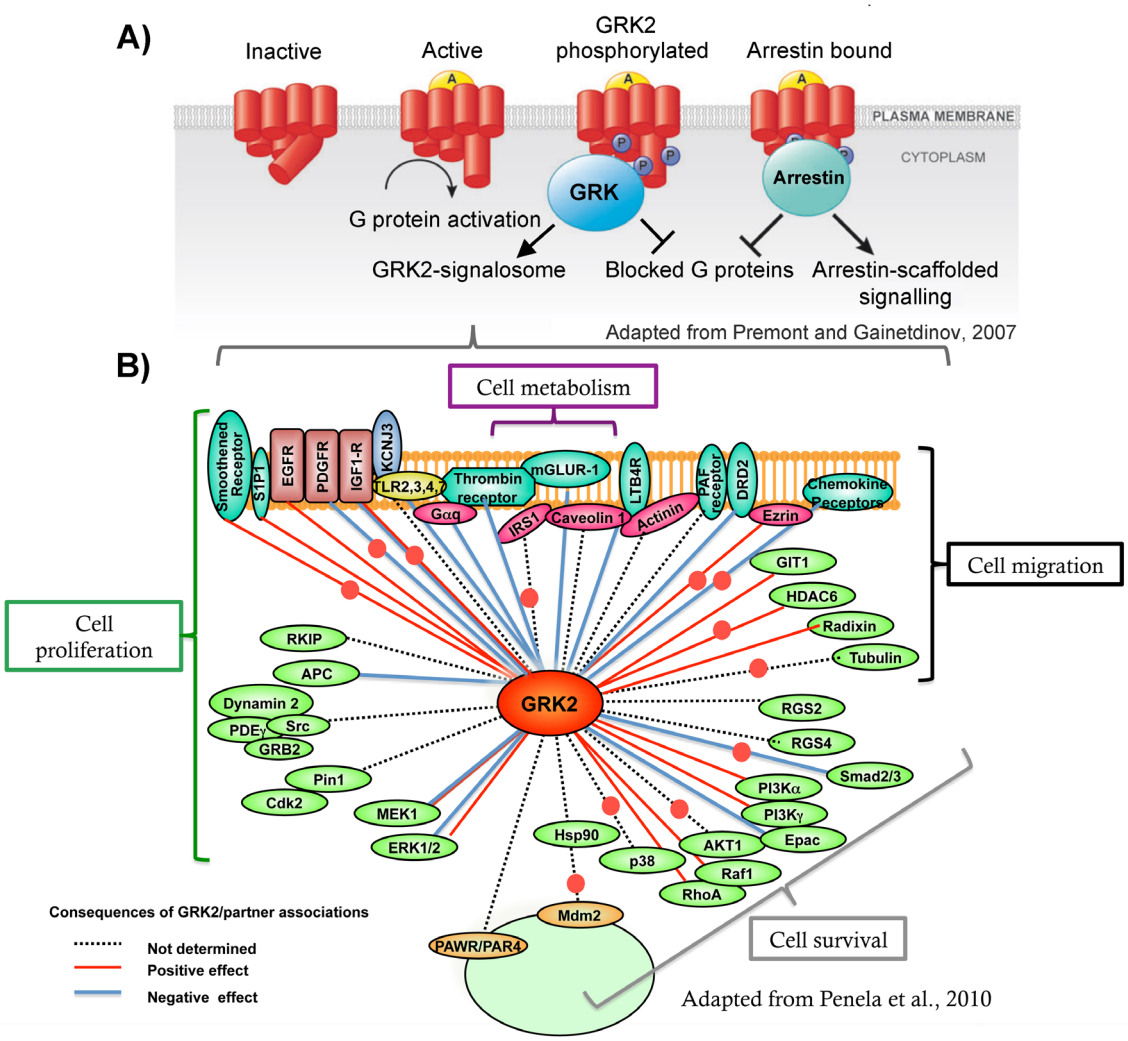


Figure I.11 The complex GRK2 interactome. In addition to its 'classical' role triggering GPCR phosphorylation and β-arrestin binding (A) , GRK2 can modulate cell signalling by interacting with and / or phosphoryating diverse non-GPCR substrates (B) which point at this kinase as a a central signalling node and key modulator of relevant biological processes such as cell proliferation, survival, migration and cell metabolism. Red circles denotes phosphorylation by GRK.

GRK2 contributes to **cell proliferation** triggered by some tumour-related GPCRs, such as the lipid S1P1 receptor, the Smoothened receptor or the chemokine receptor CXCR7 by mediating MAPK activation (Lipfert et al., 2013; Meloni et al., 2006; Molnar et al., 2007). The mitogenic effect of GRK2 is based on its ability to phosphorylate or dynamically interact (in a kinase activity-independent way) with important modulators/ efectors engaged along the MAPK pathway, such as GIT-1, Raf, RhoA, Epac, PDEγ,

RKIP or Pin1 (Deiss et al., 2012; Eijkelkamp et al., 2010; Penela et al., 2008, 2010b; Robinson & Pitcher, 2013; Wan et al., 2003). In addition, we and others have recently reported that GRK2 is necessary to ensure a proper and timely progression of cell cycle, particularly during G1/S and G2/M transitions in response to extrinsic and intrinsic cues, respectively (revised in Penela et al., 2010). GRK2 levels fluctuate along cell cycle, being down-regulated during the G2/M transition. This transient GRK2 degradation is required for normal cell cycle progression. Interestingly, the default GRK2 protein decay in G2 is prevented in the presence of DNA damaging agents that trigger cell cycle arrest such as doxorubicin. Moreover, such accumulation of GKR2 inversely correlated with the activation of p53 triggered by G2/M checkpoints mechanisms helping to restrict the apoptotic face of arrested cells (Penela et al., 2010). Accordingly, we hypothesized that GRK2 can allow cells to cope with genotoxic stress by means of potentiating cell cycle arrest protection and survival response. Moreover, a role for GRK2 in cell proliferation during early embryonic development has also been suggested (Jiang et al., 2009). The ability of GRK2 to interact with Patched and to relieve the Patched-induced cytosolic retention of cyclin B in response to Hedgehog ligand appears to underlie the stimulatory effect of GRK2 in cell division.

GRK2 is also able to interact with some key players in the processes of cellular stress response and growth arrest such as p38, Smad2/3, PI3K, AKT or Hsp90 (reviewed in Penela et al., 2010a), what might positively impact on **cell survival and resisting cell death**. p38, a critical player in apoptosis or survival in a cell-type specific context and a mediator of p53 activation in response to different stresses (Bulavin & Fornace, 2004), is phosphorylated by GRK2, what prevents binding of the upstream activator MKK6 (Peregrin et al., 2006). GRK2 also interacts with both PI3K and AKT proteins, although the functional outcome of such interactions is not straightforward, as positive and negative effects have been described in a cell type-specific context. GRK2 contributes to the GPCR-induced AKT activation by means of its association with the catalytic subunit of PI3Kgamma , while a direct interaction with the regulatory subunit p85 facilitates AKT activation in the context of cell cycle of epithelial cells (Rivas V et al., unpublished results). On the contrary, in non-epithelial cells a GRK2-mediated inhibition of Akt phosphorylation and canonical activation has been shown. Similarly, the connexion of GRK2 with the TGFβ signalling axis is also complex, as TGFbeta elicits paradoxical effects on cell proliferation and migration in a cellular context-dependent manner (Lebrun, 2012; Siegel & Massagué, 2003). In response to TGFβ-bound ALK5, GRK2 can associate to and phosphorylate Smad2/3 in their regulatory linker domain, preventing activation and nuclear translocation of the Smad complex, thereby leading to the inhibition of pro-arresting and pro-apoptotic

TGF-beta effects (Ho et al., 2005, 2007). Such regulation might favour a potential TGFβ switch from a tumour suppressor to a tumour promoter, akin to that induced by oncogenic HRas-dependent, JNK-mediated phosphorylation of Smad3 (Liu et al., 2009).

Recently, GRK2 has been shown to be involved in the **homeostasis of cellular metabolism** via the regulation of insulin and insulin-like growth factor pathways and mitochondria functionality, suggesting a novel role of GRK2 in controlling the cellular use of glucose and the ability of the cell to control energy production and expenditure (Ciccarelli et al., 2012; Vila-Bedmar et al., 2012). GRK2 over-expression inhibits glucose uptake through kinase activity-independent quenching of Gαq subunits that are required for GLUT4 translocation upon insulin stimulation, and of IRS1 transducers needed for insulin receptor downstream signalling (Garcia-guerra et al., 2010; Usui et al., 2005). Therefore, GRK2 downregulation leads to increased insulin sensitivity both in cells and in animal models of insulin resistance (Garcia-guerra et al., 2010; Gurevich et al., 2012). Interestingly, epidemiologic data suggest that patients with insulin resistance have a higher risk of developing several types of cancer, including breast cancer, as hyperinsulinemia results in stimulated DNA synthesis, cell proliferation and genomic instability. In addition, GRK2 can localize to the mitochondrial outer membrane by means of the cellular stress–induced MAPK-mediated modification of the protein and the interaction with Hsp90. The consequences of such mitochondrial translocation are controversial because both detrimental (increased cytochrome C release and apoptosis) and protective (increased biogenesis and ATP production) effects have been reported (Fusco et al., 2012; Huang et al., 2014).

Finally, compelling evidences indicate a relevant role of GRK2 in **cellular motility** (revised in (Penela et al., 2014a)). GRK2 interacts with or phosphorylates some cytoskeletal substrates implicated in cell remodelling, movement and migration such as ezrin, radixin or tubulin (Cant & Pitcher, 2005a; Kahsai et al., 2010a; Pitcher et al., 1998) (Fig. I.11), all of them involved in the acquisition of invasive properties. GRK2 phosphorylates ezrin at a single Thr567 residue in a PIP2-and Gβγ- dependent manner, which is important for maintaining ezrin in an active conformation with both the plasma membrane and F-actin binding domains accessible (Cant & Pitcher, 2005b). Similarly, GRK2 phosphorylates radixin at the critical Thr564 residue (Kahsai et al., 2010b). The functionality of the phosphorylation of tubulin by GRK2 has not been already addressed. Furthermore, an important function of GRK2 in epithelial cell migration through the interaction of GRK2 with GIT1 (Penela et al., 2008) and more recently of HDAC6 (Lafarga et al., 2012a) have been reported.

Notably, HDAC6 is a cytosolic histone deacetylase type II protein that is over-expressed in a high proportion of breast tumours and contributes to cell growth, cell motility and (interestingly) to the invadopodia formation and maturation, through the regulation of acteylation-deacetylation of a growing number of proteins including Hsp90, the DNA mismatch repair protein MSH2, β-catenin or Ras, among others (Duong et al., 2008; Lee et al., 2008; Zhang et al., 2014). Related to cell motility, tubulin and cortactin have been identified as a major HDAC6 substrates both in normal and in cancer cells (Boyault et al., 2007; Gao et al., 2007; Zhang et al., 2003). In particular, the extent of tubulin deacetylation correlates with increased microtubule dynamicity and plasticity, which are both required for cell cycle division (mitotic spindle), regulation of intracellular protein trafficking, cell motility and focal adhesion turnover (Aldana-Masangkay & Sakamoto, 2011). EGF-induced GRK2-mediated phosphorylation of HDAC6 in residues serine 1060/1062 and 1068 is necessary for both its full tubulin-deacetylation activity and pro-migratory effects (Lafarga et al., 2012).

In sum, it is reasonable to hypothesize that through this complex interactome, GRK2 may act as an onco-modifier and assist in tumoural transformation in a cell-specific manner.

c. Regulation of GRK2

Due to the complexity of GRK2 and its interactome, this protein is subject to a tightly regulation of its expression levels, activity and subcellular location. In this sense, our group has focused over the past decade on the study of the different mechanisms of GRK2 modulation, particularly of those related to the control of GRK2 degradation.

GRK2 protein interacts with different regulators such as calcium-binding proteins, alpha-actinin, Hsp90 or caveolin, and undergoes different types of postranslational modifications that can either regulate its catalytic activity, divert the kinase to different substrates or modulate its subcellular location or proteolysis rate (reviewed in (Ribas et al., 2007)). Kinase activity towards GPCRs is inhibited by the S-nitrosylation of Cys residues within its catalytic domain (Whalen et al., 2007). Second messenger-modulated kinases PKC and PKA can phosphorylate GRK2, leading to positive modulation of kinase activity and membrane targeting, respectively. In addition, non-receptor tyrosine kinases such as c-Src and Proline-directed kinases (MAPK and CDK2-cyclinA) target the GRK2 protein. The activity of tyrosine-phosphorylated GRK2 is increased toward both soluble and membrane bound substrates, suggesting a direct effect on its catalytic activity, while modification of S670 by ERK1-2 or CDK2 hampers the βγ-mediated recruitment of GRK2 to the plasma membrane (Elorza et al., 2000). Interestingly, this latter modification at the C-terminus of

GRK2 causes a switch in the repertoire of kinase substrates, as it promotes the acquisition of a distinctive competent conformation at the active site (and not in docking sites) that allow phosphorylation of HDAC6 (Lafarga et al., 2012b). Since allosteric communication has been suggested to take place between the C-tail PH domain and the RH domain of GRK2, it is possible that structural alterations caused by covalent modifications at the C-terminus could be transmitted to the catalytic domain, resulting in different conformations.

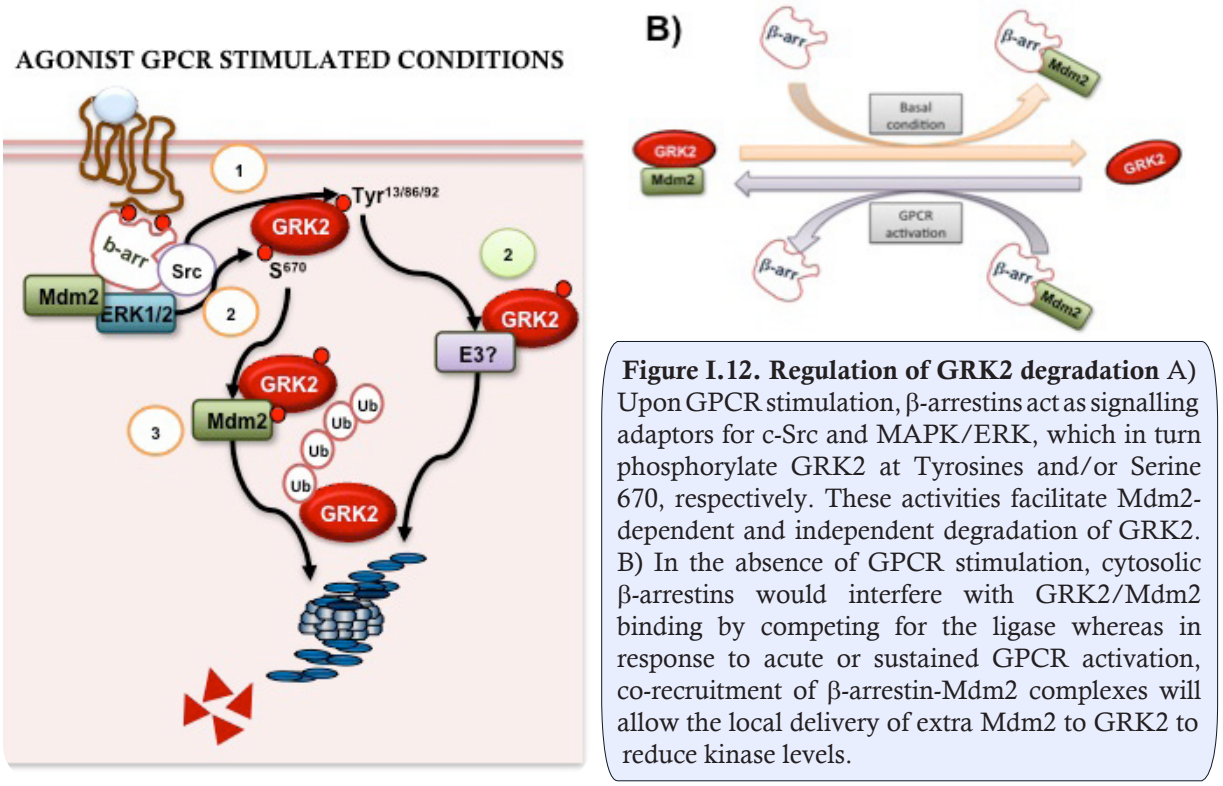


Figure I.12. Regulation of GRK2 degradation A) Upon GPCR stimulation, β-arrestins act as signalling adaptors for c-Src and MAPK/ERK, which in turn phosphorylate GRK2 at Tyrosines and/or Serine 670, respectively. These activities facilitate Mdm2-dependent and independent degradation of GRK2. B) In the absence of GPCR stimulation, cytosolic β-arrestins would interfere with GRK2/Mdm2 binding by competing for the ligase whereas in response to acute or sustained GPCR activation, co-recruitment of β-arrestin-Mdm2 complexes will allow the local delivery of extra Mdm2 to GRK2 to reduce kinase levels.

On the other hand, phosphorylation has also direct effects on GRK2 stability (Fig.I 12A). Upon GPCR activation GRK2 is rapidly degraded in a ubiquitin and proteasome-dependent manner (Penela, 1998). Further studies revealed that both c-Src and MAPK-dependent phosphorylation of GRK2 underpin GPCR-induced degradation (Carman et al., 1999; Elorza et al., 2003; Penela et al., 2001). c-Src phosphorylation sites have been localized to Tyr13, 86, and 92, and the Y13/86/92F GRK2 mutant is resistant to degradation (Penela et al., 2001). Although both c-Src- and ERK-mediated phosphorylation of GRK2 could target GRK2 for degradation independently, ERK preferentially phosphorylates GRK2 previously phosphorylated on tyrosine residues by c-Src (Elorza et al., 2003). Interestingly, our group discovered Mdm2 as the main E3 ligase implicated in GRK2 turnover by the proteasome pathway (Salcedo et al., 2006). Recently, we further characterized the Mdm2-dependent GRK2 degradation and found that, upon GPCR activation, the Mdm2 action on GRK2 is dependent on the previous phosphorylation of GRK2 at Ser670 by MAP kinases, while tyrosine phosphorylation was dispensable (Nogués et al., 2011).

β-arrestins are known to recruit c-Src, ERK and Mdm2 to the vicinity of activated GPCRs (Luttrell et al., 2001; Wang et al., 2003) and are the scaffolding molecules in charge of facilitating their interaction with GRK2 upon GPCR activation. However, in the absence of GPCR activity, arrestins do not participate in the Mdm2-mediated regulation of the GRK2 basal turn-over, but instead compete with GRK2 for Mdm2 and suppress basal GRK2 degradation (Nogués et al., 2011). In basal conditions, c-Src is also able to mediate Mmd2-independent GRK2 degradation, although the E3 ligase implicated in this process remains to be elucidated. Thus, arrestins play a coordinating role recruiting kinases and/or ubiquitin ligases to GRK2 in the basal condition or upon activation of GPCRs, regulating GRK2 turnover via different pathways (Fig.I.12).

As aforementioned, GRK2 expression levels are also tightly controlled during cell cycle progression. Phosphorylation of the GRK2 S670 residue by the cell-cycle kinase CDK2-Cyclin A and the subsequent binding of the prolyl-isomerase Pin1, are required for transient GRK2 degradation during the G2/M transition (Penela et al., 2010b). Mdm2 was shown to be the main E3 ligase implicated in the CDK2-Pin1-dependent degradation of GRK2 (in a way independent of the beta-arrestin scaffolding function), although the contribution of other ligases to this process cannot be ruled out. Finally, interaction of GRK2 with the heat shock protein Hsp90 is also involved in the maintenance of stable kinase levels in cells. GRK2 - Hsp90 interaction seems to play a role in the proper folding and maturation of the newly synthesized GRK2 (Luo & Benovic, 2003).

In contrast to the well-known regulation of GRK2 by post-transcriptional modifications and the proteasome pathway, little is known about the regulation of GRK transcription or mRNA translation. The GRK2 promoter was considered to be typical of ‘‘housekeeping’’ genes. Studies using the human GRK2 gene promoter in aortic smooth muscle cells have revealed that its transcriptional activity is increased by phorphol esters or activation of Gαq or alpha1-adrenergic signalling pathways. On the contrary, pro-inflammatory cytokines promoted the opposite effect (Penela et al., 2003).

Despite the fact that GRK2 is very finely regulated by the different mechanisms, summarized above, the balance between them seems to be frequently altered in pathological conditions, leading to alterations in GRK2 levels or activity. Consistently, aberrant GRK2 functionality is found in inflammatory pathologies, metabolic or cardiovascular diseases and certain tumours, such as in granulose cell tumours, thyroid and prostate cancer (Métayé et al., 2002; King et al., 2003; Prowatke et al., 2007; Penela et al., 2010), what suggests a potential role for GRK2 in the triggering or development of such processes.

Regarding cancer, we have reported the up-regulation of GRK2 in different malignant mammary cell lines with aberrant activation of the PI3K/AKT pathway (Salcedo et al., 2006)(Fig.I.13). The activation of the PI3K/Akt pathway by agonists such as IGF-1 alters Mdm2 phosphorylation and triggers its nuclear localization, thus hampering Mdm2-mediated GRK2 degradation, leading to enhanced GRK2 protein levels. Since Mdm2 over-expression or its functional amplification are frequent events in neoplastic diseases, it's feasible that in some tumoural contexts the increase in GRK2 expression might correlate with a higher activity of the ligase. Such circumstance may reflect a functional cooperation between GRK2 and Mdm2 in order to confer resistance to growth arrest and to apoptosis, as suggested by the fact that GRK2 attenuates the DNA damage-induced increase of p53, acting either in parallel to or in line with Mdm2 (Penela et al., 2010). In agreement with these hypotheses, in this work we have intended to investigate the impact of GRK2 expression in the tumoural hallmarks of breast cancer cells, and to delineate the functional consequences of altered GRK2 levels on the Mdm2 regulatory properties, with emphasis in the p53/Mdm2 regulatory axis .

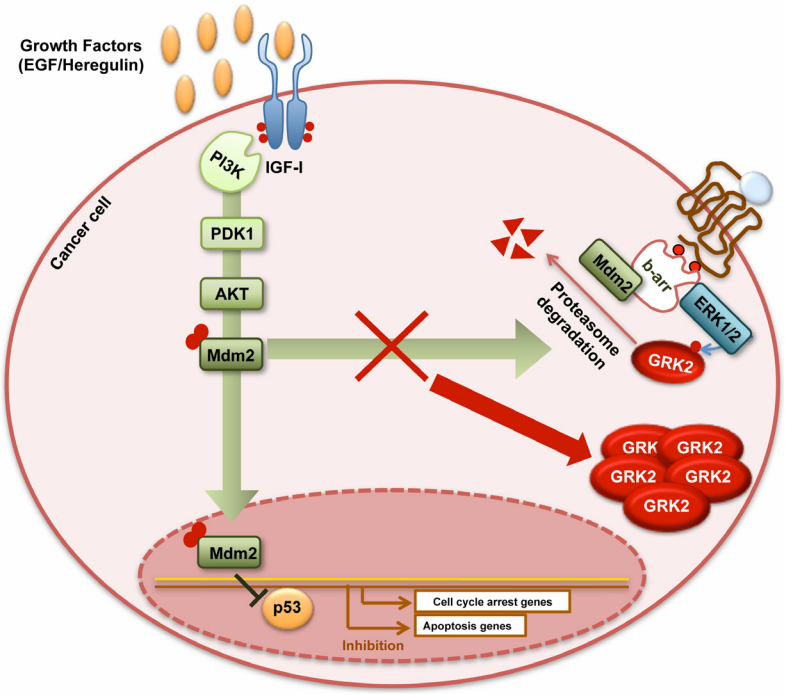


Figure I.13. Tumoural de-regulation of GRK2 turnover. IGF-1 stimulation induces GRK2 protein accumulation in a PI3K-dependent manner, by relieving the Mdm2- dependent degradation of GRK2.

OBJECTIVES

Despite of its profuse connectivity to diverse signalling pathways that impact on cell transformation, and the high-level expression of GRK2 in some tumoural contexts, a comprehensive study of the role of this kinase in tumour formation and progression has not been addressed in detail. Contradictory results on its effects on cell proliferation have been reported depending on the tumour type. However, most of these studies relayed on the use of cellular models and focused on "in vitro" cell-culture proliferation, and did not address whether GRK2 might influence in vivo tumour proliferation or other processes relevant to tumour biology, such as the anchorage-independent growth or the cellular survival to the stressful tumoural milieu. Therefore, the purpose of this research work was to identify and analyze in deep the contribution of GRK2 to breast cancer tumorigenesis, paying special attention to the unexplored role of this protein in breast cancer proliferation and invasion and its connection with the main oncogenic pathways involved in this type of cancer. Moreover, the possible connection between GRK2 and Mdm2 or HDAC6 in a breast cancer context was specifically investigated.

Specific objectives:

1. Investigate the occurrence of altered GRK2 levels in breast cancer, their underlying causes and the functional interplay between the upregulation of the oncoprotein Mdm2 and GRK2 levels.

2. Analyze the regulatory loop between Mdm2 and GRK2 and delineate the functional consequences of altered GRK2 levels in the Mdm2/p53 regulatory axis.

3. Determine the implication of GRK2-dependent regulation of HDAC6 in cell transformation and its relevance in wild-type or mutated p53 backgrounds.

4. Identify the impact of altering GRK2 levels in the acquisition of hallmarks of breast cancer, with particular focus on cell proliferation, survival and invasion.

MATERIALS & METHODS

1.

Materials

1.1. Buffers and solutions

Name	Composition	Comments
Buffer C	20 mM HEPES pH 7,5 0.45 M NaCl 1 mM EDTA	Add fresh a protease inhibitor cocktail
Coomasie staining solution	0.1%(w/v) Coomassie R250 10% (v/v) acetic acid 50% (v/v) methanol 40% (v/v) H_2O	
Coomasie destaining solution	10% (v/v) acetic acid 50% (v/v) methanol 40% (v/v) H_2O	
Hypotonic buffer	20 mM HEPES pH 7,5 10 mM $MgCl_2$ 1 mM NaF 0,1 mM EDTA 1mM DTT	

Kinase assay buffer	27 mM Tris-HCl (pH 7.5) 6 mM MgCl2 7,5 mM NaF 1 mM EGTA 1,4 mM EDTA 10 µM ATP [γ32P]-ATP (2-3 cpm/pmol)	 (Perkin Elmer)
LB broth	1% (w/v) bacto-tryptone 0.5% (w/v) bacto-yeast extract 1% (w/v) NaCl (171 mM)	autoclaved
LB-agar	LB broth + 1.5% (w/v) agar	autoclaved
Mdm2-p53 interaction buffer	25 mM Tris-HCl (pH 8) 2.7 mM KCl 137 mM NaCl 10%(v/v) Triton-X100 10% Glicerol	Add fresh a protease inhibitor cocktail
PBS (1x)	137 mM NaCl (0.8% w/v) 2.7 mM KCl (0.02% w/v) 10 mM Na_2HPO_4 (0.144% w/v) 1.75 mM KH_2PO_4 (0.024% w/v) pH 7.4	autoclaved
Ponceau staining solution	0.2% Ponceau-S in 3.0% TCA	
Protease/phosphatase Inhibitor Cocktail:	0.1 µM PMSF 0.5 µM Benzamidin 1 µg/µl Aprotinin 0.1 µM $NaVO_3$ 10 µg/ml STI 10 µg/ml Bacitracin	
Protein sample buffer 4x	200mM Tris pH=6.8 40% (v/v) glycerol 0.15% (w/v) bromophenol blue 4% (w/v) SDS 5% (v/v) b-Mercaptoethanol	
RIPA buffer (1x)	Tris-HCl 20mM pH 7.5 150mM NaCl 1% Triton-X100 0.1% SDS 0.5% sodium deoxycholate	store at 4°C for less than 3 months Add fresh a protease inhibitor cocktail

Resolving Polyacrylamide gel (6-15%)	Acrylamide/N,N'methylen-bisacrylamide 30:0.8 (w/v) 0.375 M Tris-HCl pH 8.9 0.1% SDS	
Running buffer(10x)	0.25 M Tris base 2.0 M glycine 1% SDS	
Stacking Polyacrylamide gel (4%)	Acrylamide/N,N'methylen-bisacrylamide 30:0.8 (w/v) 0.25 M Tris-HCl pH 6.8 0.1% SDS	
TAE buffer (1x)	40 mM Tris-acetate pH 7.6 1 mM EDTA	autoclaved
TBS (10x)	0.1 M Tris base 1.5 M NaCl pH 7.4 with HCl conc.	
TBS-Tween (10x)	0.1 M Tris base 1.5 M NaCl 0.2% Tween-20 pH 8.0 with HCl conc.	
Transfer buffer (2l)	0,64g Na_2CO_3 1,68g $NaHCO_3$ 20% methanol (v/v)	

1. 2. Oligonucleotides

All oligonucleotides for mutations were purchased from Sigma-Aldrich Spain

Primer name	**Oligonucleotide sequence**
Flag-Mdm2 S115/116A Fw	5′ - cagcaggaagcagcggactcaggtac - 3′
Flag-Mdm2 S115/116A Rev	5′ - gtacctgagtccgctgcttcctgctg - 3′
Flag-Mdm2 S172A Fw	5′ - cagaagaaaatgcagatgaattatctgg - 3′
Flag-Mdm2 S172A Rev	5' - ccagataattcatctgcattttcttctg - 3'
Flag-Mdm2 S213/216/218A Fw	5' - gaagcagtagcgctgaatctgcaggggcgccatcgaatc - 3'
Flag-Mdm2 S213/216/218A Rev	5' - gattcgatggcgcccctgcagattcagcgctactgcttc - 3'
Flag-Mdm2 S254/256A Fw	5' - gaagttgaagctctcgacgcagaagattatagcc - 3'
Flag-Mdm2 S254/256A Rev	5' - ggctataatcttctgcgtcgagagcttcaacttc - 3'
Flag-Mdm2 S260/262A Fw	5' - cgactcagaagattatgcccttgctgaagaagg - 3'

Flag-Mdm2 S284/286A Fw	5' - ggcaggggaggctgatacagattc - 3'
Flag-Mdm2 S284/286A Rev	5' - gaatctgtatcagcctcccctgcc - 3'
Flag-Mdm2 T350/351A Fw	5' - ctggaaaacgcagcacaagctgaagagg - 3'
Flag-Mdm2 T350/351A Rev	5' - cctcttcagcttgtgctgcgttttccag - 3'
Quickchange - pDest - Generation	
HDAC6 - Fw	5' - caccatgacctcaaccggcc - 3'
GFP- Rev	5' - gggttacttgtacagctcgtccatgccgac - 3'

1. 3. DNA enzymes

Enzyme	Supplier
BP Clonase	Invitrogen
DpnI	New England Biolabs
LR Clonase II	Invitrogen
Pfu ultra	Stratagene
Platinum Taq	Invitrogen
Taq polymerase	New England Biolabs

1. 4. Primary antibodies

Antigen	Reference	Supplier	Species	Dilution
Acetyl-a-tubulin	Clone 6-11B-1	Sigma	Mouse	WB (1/2000) / IF (1/100)
Actin	I-19 (sc-1616)	Santa Cruz	Goat	WB (1/1000)
Akt	9272	Cell Signalling	Rabbit	WB (1/1000)
Cleaved-caspase 3	Asp175	Cell Signalling	Rabbit	WB (1/1000) / IHC (1/200)
Cortactin	Clone 4F11	Millipore	Mouse	WB (1/1000) / IF (1/200)
Cyclin D1	sc246	Santa Cruz	Mouse	WB (1/1000)
E-Cadherin	4065	Cell Signalling	Rabbit	WB (1/1000)
Erk1	(C-16): sc-93	Santa Cruz	Rabbit	WB (0.75 mg/ml)
Erk2	(c-14): sc-154	Santa Cruz	Rabbit	WB (0.75 mg/ml)
Falloidin-TRITC	P-1951	Sigma	-	IF (1/100-1/500)
FLAG M1	F3040	Sigma	Mouse	WB (1:1000) / IP (1-2 mg)
GAPDH	ab8245	Abcam	Mouse	WB (1:2000 - 1/4000)
GIT1	H-170 (sc-13961)	Santa Cruz	Rabbit	WB (1/500)
GRK2	C-15 (sc-562	Santa Cruz	Rabbit	WB (1/500)
GRK2/3	clone C5/1.1 05-465	Millipore	Mouse	WB (1:2000) / IP (1-2 mg)
GRK2 PF1		Our laboratory	Rabbit	WB (1:1000) / IP (1-2 mg)
GRK2 PF2		Our laboratory	Rabbit	IHC (1/200-1/1000)
GST	(Z-5): sc-459	Santa Cruz	Rabbit	WB (1/500)
HDAC6	H-300	Santa Cruz	Rabbit	WB (1/500)
KI67	Clone SP6	Thermo Scientific	Rabbit	IHC (1/300)

Mdm2	N-20 (sc-813)	Santa Cruz	Rabbit	WB (1/500) / IP (1-2mg)
Mdm2	Ab-5 (4B2C1.11)	Calbiochem	Mouse	WB (2 mg/ml)
Mdm2	SMP-14 (sc-965)	Santa Cruz	Mouse	WB (1/500)
Mdm2	SMP14	DAKO	Mouse	IHC (1/200)
MT1-MMP		Dr. P.Chavrier	Mouse	WB (1/1000)
Noxa	114C307	Abcam	Mouse	WB (1-2 mg/ml)
Nucleolin	CL3(H-250) (sc-13057)	Santa Cruz	Rabbit	WB (1/1000)
p21	C-19 (sc-397)	Santa Cruz	Rabbit	WB (1/1000)
p53	DO-1 (sc-126)	Santa Cruz	Mouse	WB (1/1000) / IHC (1/400)
p-Akt (Ser473)	9271	Cell Signalling	Rabbit	WB (1/1000) / IHC (1/200)
p-Erk1-2 (Thr202/204)	9101	Cell Signalling	Rabbit	WB (1/1000)
p-GRK2 (Ser670)	44202	Biosource	Rabbit	WB (1/500)
p-Histone-3 (Ser10)	06-570	Millipore	Rabbit	WB (1/500)
p-p53 (Ser15)	16G8 (9286)	Cell Signalling	Mouse	WB (1/500)
Pan-Ras	Ab-3	Calbiochem	Mouse	WB (2.5 mg/ml)
Pin1	C-20 (sc-7409)	Santa Cruz	Rabbit	WB (1/1000)
Pro-Caspase 3	9662	Cell Signalling	Rabbit	WB (1/1000)
a -Tubulin	DM1A	Sigma	Mouse	WB (1/1000)
Vimentin	Clone V9	Dako	Mouse	WB (1/1000)

1. 5. Antibodies conjugated to agarose beads

Antigen	Epitope/Reference	Supplier	Species	Dilution
P53-AC	DO-1	Santa Cruz	Mouse	IP (2 mg/sample)
Mdm2-AC	SMP-14	Santa Cruz	Mouse	IP (1-2mg/sample)

1. 6. Secondary antibodies

Name, antigen	Company	Dilution
Alexa Fluor 680, goat anti-mouse IgG	Molecular probes	1 mg/ml
IRDye™ 800 CW, donkey anti-mouse IgG	Rockland	0.5 mg/ml
Alexa Fluor 680, goat anti-rabbit IgG	Molecular probes	1 mg/ml
IRDye™ 800 CW, goat anti-rabbit IgG	Rockland	0.5 mg/ml
Alexa Fluor 680, donkey anti-goat IgG	Molecular probes	1 mg/ml
IRDye™ 800 CW, goat anti-goat IgG	Rockland	0.5 mg/ml
Soluble rabbit peroxidase-anti-mouse IgG	Nordic immunology	1/50000
Soluble mouse peroxidase-anti-rabbit IgG	Nordic immunology	1/50000
Soluble mouse peroxidase-anti-goat IgG	Nordic immunology	1/50000
Biotin-conjugated donkey anti-rabbit IgG	Jackson Immnunoresearch	1/100 (IHC)
Biotin-conjugated donkey anti-mouse IgG	Jackson Immnunoresearch	1/100 (IHC)

1. 7. Plasmids

cDNA	vector	Origin
EGFR		
Flag-Mdm2	pCMV	Dr. L Mayo (, USA)
Flag-Mdm2 S115/116A	pCMV	Generated as part of this thesis
Flag-Mdm2 S172A	pCMV	Generated as part of this thesis
Flag-Mdm2 S213/216/218A	pCMV	Generated as part of this thesis
Flag-Mdm2 S254/256A	pCMV	Generated as part of this thesis
Flag-Mdm2 S260/262A	pCMV	Generated as part of this thesis
Flag-Mdm2 S284/286A	pCMV	Generated as part of this thesis
Flag-Mdm2 T350/351A	pCMV	Generated as part of this thesis
GRK2	pcDNA3.1	Dr J Benovic (The Kimmel Cancer Center, USA)
GRK2 K220R	pcDNA3.1	Dr J Benovic (The Kimmel Cancer Center, USA)
HDAC6-GFP	pEGF	Dr F Sanchez-Madrid (ISS La Princesa, Madrid, Spain)
HDAC6 S1060/1062/1068A-GFP	pEGF	Generated in our laboratory
Her/Neu (ERBB2)	pORF	InvivoGene
Mdm2	pC/pCDNA3	Dr. Manuel Serrano (CNIO, Spain)
p53	pLMV	Clontech
pDest-HDAC6-GFP	pDEST-DH1	Generated as part of this thesis
pDest- HDAC6 S1060/1062/1068A-GFP	pDEST-DH1	Generated as part of this thesis
pMD2G		Dr F.Martin Belmonte (CBMSO, Spain)
psPax2		Dr F.Martin Belmonte (CBMSO, Spain)
Ras-V12		Dr. Manuel Serrano (CNIO, Spain)

1. 8. Adenoviral constructs

Name	Species	Origin
Cre-recombinase		Gene Transfer Vector Core Facility, University of Iowa
GRK2	human	Provided by Dr. S. Ferguson (Robarts Research Institute, Ontario, Canada)
Sh-GRK2	rat	5'- GCAGAGACGTCTTTGATACC-3' (Generated in our Laboratory)
GRK2-K220R	human	Provided by Dr. S. Ferguson (Robarts Research Institute, Ontario, Canada)
Lamin	human	Provided by Dr. J.Aldudo (CBMSO, Spain)
Sh-GRK2	human	5′- GCAAGAAAGCCAAGAACAAGC-3′ /Generated in our Laboratory)

1. 9. Human SiRNA constructs

Name	Origin /Company
GRK2 #1 (Hs_ADRBK1_1)	Qiagen
GRK2 #2 (Hs_ADRBK1_2)	Qiagen
GRK2 #9 (Hs_ADRBK1_9)	Qiagen
GRK2 #10 (Hs_ADRBK1_10)	Qiagen
GRK2 (On target SmartPool)	Dharmacon
HDAC6 (On target SmartPool)	Dharmacon
MT1-MMP (On target SmartPool)	Dharmacon
Non-Targeting (On target SmartPool)	Dharmacon

1. 10. Fusion proteins

Name	Origin
GRK2	Generated in our laboratory
GST	Generated in our laboratory
GST-Mdm2	Generated in our laboratory
GST-Mdm2	Abnova
GST-Mdm2 100-200	Generated in our laboratory
GST-Mdm2 100-491	Generated in our laboratory
GST-Mdm2 ring domain	Generated in our laboratory
His-Mdm2	Abcam

1. 11. Cell treatments

Name	Description	Company
17-b-Estradiol	Oestrogen	Sigma
4' OHT	Tamoxifen	Sigma
AG1478	EGFR inhibitor	Sigma
CCL-21	Chemokine (signalling through the CCR7 receptor)	Preprotech
Cycloheximide	Protein synthesis inhibitor	Calbiochem
Cisplatin	Apoptosis Inductor	Sigma
Doxorubicin	Inhibitor of macromolecular biosynthesis.	Sigma
EGF	Agonist of EGFR. Acts as a survival factor	Calbiochem
Etoposide	Cytotoxic agent (topoisomerase inhibitor)	Sigma
Heregulin	Growth factor that binds with ERB3 and ERB4 receptors. It also promotes motility and invasiveness of breast cancer cells.	Preprotech
HLI737	Inhibitor of Mdm2 ubiquitin ligase (E3)	BostonBiochem
Nutlin3a	Inhibitor of Mdm2-p53 interaction	Calbiochem
Paclitaxel	Drug that interferes with the normal breakdown of microtubules during cell division	Sigma
Saha (Vorinostat)	Histone deacetylase inhibitor	Cayman chemical company
SDF1a	Chemokine (signalling through the CXCR4 receptor)	Preprotech
Tubacin	Selective HDAC6 inhibitor	Enzo

1. 12. Reagents

Name	Company
Charcoal Medium	Invitrogen
Collagen type I (rat-tail)	BD-Biosciences
DMEM	GIBCO
DAB	Vector Laboratories
Doxycyclin	Sigma
Dynabeads	Dynal
Elite ABC kit	Vector Laboratories
F12-HAMs	GIBCO
Gelatin GFP-Conjugates	Molecular Probes
Hydrocortisone	Sigma
Hygromicin	Sigma
Hyperfilms	Amersham
Insulin	GIBCO
Lipofectamine	Life-technologies
Lullaby	OZ-Biosciences
Matrigel	BD-Biosciences
Nuclear extraction kit	Panomics
Nucleofection technology	Lonza
[γ32P]-ATP	Perkin-Elmer
Protein/DNA arrays	Panomics
Puromycin	Invitrogen
Ras activity assay	Cytoskeleton
35S-labeled methionine/cysteine	New England Nuclear.
Tetracyclin	Sigma
Transferrin	Sigma

1. 13. Cell lines

Cell lines	Origin
184B5	ATCC
293T	Dr. Fernando Martin Belmonte (CBMSO)
HEK293	ATCC
Hs578T	Dr. José M. Cuezva (CBMSO, Madrid, Spain)
MCF7	Dr. Manuel Serrano (CNIO, Madrid, Spain)
MCF10A	Dr. Manuel Serrano (CNIO, Madrid, Spain)
MDA-MB-157	ATCC
MDA-MB-231	ATCC
MDA-MB-361	ATTC
MDA-MB-468	Dr. Manuel Serrano (CNIO, Madrid, Spain)
T47D	ATCC

Stable cell lines

184B5-GRK2-A	Generated in our laboratory
184B5-GRK2-R	Generated in our laboratory
MCF7-Tet-On-GRK2-wt16	Generated in our laboratory
MCF7-Tet-On-GRK2-K220R	Generated in our laboratory
MDA-MB-231-H2B-GFP	Dr. P.Chavrier (Institut Curie, Paris, France)
MDA-MB-231-HDAC-GFP	Generated as part of this thesis
MDA-MB-231-HDAC6-S1060/1062/1068A-GFP	Generated as part of this thesis

2.

Methods

2.1 DNA manipulations

a) DNA mutagenesis

The possible specific sites of Mdm2 phosphorylation by GRK2 were mutated using the QuickChange® site-directed mutagenesis kit (Stratagene). The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest (Flag-Mdm2) and two synthetic oligonucleotide primers, each complementary to opposite strands of the insert sequence containing the desired point mutations. The primers were designed according to the manufacturer's guidelines and extended by PCR using the reagents provided in the kit under the following conditions:

Step	Cycles	Temperature	Time	Purpose
1	1	95ºC	30 sec	
2	18	95ºC	50 sec	DNA denaturing
		60ºC	50 sec	Primers annealing
		68ºC	7 min	Template elongation
3	1	68ºC	7 min	
4	1	4ºC	∞	

Following the PCR, products were treated with DpnI restriction enzyme for 1 hour at 37°C to digest the parental DNA template. To select for mutation-containing synthesized DNA, E.coli XL1-Blue supercompetent cells were transformed with the resulting nicked vectors according to manufacturer's instructions and bacteria plated onto LB-ampicillin (50 μg/ml) agar plates. Resulting colonies were grown in LB broth-ampicillin (10 μg/ml) for plasmid preparation (Promega Spin Miniprep Kit, Promega). Plasmids were subjected to DNA sequencing to verify the mutation and the fidelity of the whole cDNA sequence.

Finally, Large-scale purification of plasmid DNA (maxi-prep) was performed using Endofree Plasmid purification kit® (Qiagen) according to the manufacturer's instructions, and plasmids were transfected into 293 cells to check the proper expression of the mutants. (Fig. MM.1).

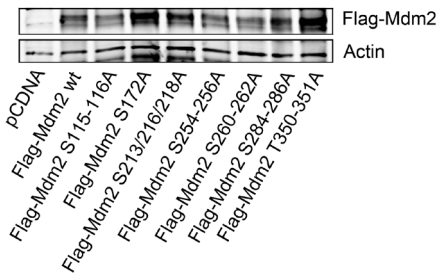


Figure MM.1: Generation of possible GRK2-dependent phosphorylation defective mutants in Mdm2. Mutants were designed as described above and, once they had been sequenced and amplified, plasmids were transfected into 293 cells. A representative western blot of the different mutants generated is shown.

b) Generation of retroviral constructions

HDAC6-GFP and HDAC6-S1060/1062/1068A-GFP pDEST (lentivirus) constructs were generated using the Gateway® system of invitrogen. The Gateway® Technology is a universal cloning method that uses the lambda recombination system to facilitate transfer of heterologous DNA sequences (flanked by modified att sites) between vectors (Hartley, 2000, Landy, 1989). The two recombination reactions that constitute the basis of the Gateway® Technology are briefly summarized in fig. MM.2.

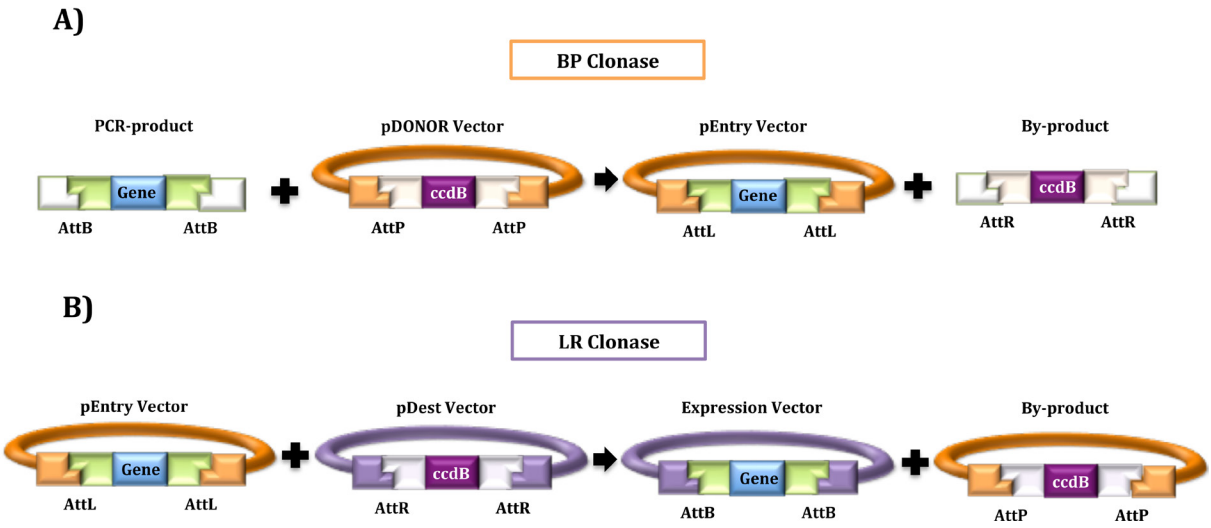


Figure MM.2: The basis of the Gateway System. A) BP Reaction: Facilitates recombination of an attB substrate (attB-PCR product or a linearized attB expression clone) with an attP substrate (donor vector) to create an attL-containing entry clone. This reaction is catalyzed by BP Clonase™ enzyme mix. B) LR Reaction: Facilitates recombination of an attL substrate (entry clone) with an attR substrate (destination vector) to create an attB-containing expression clone. This reaction is catalyzed by LR Clonase™ enzyme mix.

Briefly, the basic procedure requires the cloning of a blunt-end PCR product into one of the pENTR™ TOPO® vectors to generate an entry clone. Afterwards, performing an LR recombination reaction, an expression construct® destination vector of choice (lentivirus) was generated, and finally the expression construct is introduced into the appropriate host (293T cells) to generate the lentivirus and infect the desired cells to express the proteins.

First of all, we used the pENTR™ Directional TOPO®Cloning Kits to directionally clone a blunt-end PCR product into a vector for entry into the Gateway® System. In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC) at the 5′ end of the primer. The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. To ensure that our PCR product clones directionally with high efficiency, the reverse PCR primer must not be complementary to the overhang sequence GTGG at the 5′ end.

The primers HDAC6-Fw and GST-Rv were extended by PCR using dsDNA of HDAC6 constructs as template, the reagents provided in the kit and the Quick Change XL-Site-Directed PCR under the following conditions:

Step	Cycles	Temperature	Time	Purpose
1	1	95°C	30 sec	
2	18	95°C	50 sec	DNA denaturing
		60°C	50 sec	Primers annealing
		68°C	7 min	Template elongation
3	1	68°C	7 min	
4	1	4°C	∞	

Blunt-end PCR products were verified by using agarose gel electrophoresis (1%) and gel-purify by using the Qiagen gel extraction kit (Qiagen). Purified products were cloned into the pENTR™ TOPO® vector following the manufacturer's indications. One Shot® competent E. coli cells were transformed with the resulting recombinant vectors and plated onto LB-kanamicin (50 μg/ml) agar plates. Upon plasmid preparation (Endofree Plasmid purification kit®, Qiagen), positive colonies were confirmed by an additional PCR using the primers HDAC6-Fw and GST-Rv under the following conditions.

After selection and sequencing of the pENTRYs, the positive plasmids were subjected to a LR recombination reaction (Gateway® LR Clonase® II enzyme mix) between an attL-containing entry clone and an attR-containing destination vector to generate an expression clone (pDEST DH1= lentivirus) containing our proteins of interest. The LR recombination reaction was performed as indicated by the manufacturer.

Reactions were incubated overnight at room temperature and stopped by adding 2 μl of the Proteinase K solution and incubating them for 10 minutes at 37°C. pDEST® constructs were then transformed into DH5α™competent E. coli and plated onto LB-ampicilin (50 μg/ml) agar plates. Resulting colonies were grown in LB broth- ampicilin (50 μg/ml) for plasmid preparation (Endofree Plasmid purification kit®, Qiagen). Plasmids were subjected to DNA sequencing to verify the fidelity of the whole cDNA sequence.

c) Transformation of electrocompetent bacteria

50 μl of electrocompetent E. coli (XL1-Blue supercompetent cells, Stratagene, One Shot® TOP10 cells or electrocompetent DH5a cells) were transformed with 10 ng of plasmid following the well-established Heat-shock bacterial transformation protocol.

d) Storage of bacteria

The long-term storage of bacteria containing transformed plasmids was at -80 °C in 10% glycerol.

e) DNA sequencing

Isolated plasmids were sequenced by the external sequencing facility (Parque Científico de Madrid) using specific primers to verify the mutation and the fidelity of the whole cDNA sequence. The chromatograms were analysed with the Chromas Lite 2.1 software and fasta files were analysed with Sequence Viewer software to annotate the final sequence and to perform alignments of DNA sequences.

f) DNA agarose gel electrophoresis

Size determination and separation of DNA was achieved by means of TAE agarose gel electrophoresis. DNA samples were run in 0.8-1% (w/v) of electrophoresis grade agarose (Invitrogen) gels supplemented with0.6 μg/ml of ethidium bromide. As DNA size markers HindIII digestion fragments of λ and ø29 phages were used. Gels were run at 0.01 Volts/mm2 and DNA was visualised using a UV transilluminator.

g) Quantification of DNA

The concentration of DNA samples was measured with a Nanodrop spectrophotometer and software (Thermo Scientific). Quantification of the band obtained in the 1% (w/v) agarose gel was also used to measure DNA concentration.

2. 2 Culture and manipulation of mammalian cell lines

a) Maintaining and subculturing the cells

184B5 and MCF10A cells were cultured in DMEM /F12-HAMS medium supplemented with glutamine 2mM and penicillin/streptomycin (0.01%/0.063%) and containing 5% (v/v) horse serum (HS), 10 μg/ml insulin, 20 ng/ml EGF, 100 μg/ml transferrin, and 0.5 μg/ml hydrocortisone. MDA-MB-361, MDA-MB-468, MCF7 and Hs578T cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), glutamine 2mM and penicillin/streptomycin (0.01%/0.063%) at 37°C in a humidified 5% CO2 atmosphere. T47D cells were cultured in DMEM 10% FBS supplemented with 0.2 Units/ml bovine insulin, glutamine 2mM and penicillin/streptomycin (0.01%/0.063%). MDA-MB-157 and MDA-MB-231 cells from ATTC were first cultured and amplified in L-15 medium supplemented with glutamine 2mM and penicillin/streptomycin (0.01%/0.063%) and containing 15% (v/v) FBS at 37% in an CO2 null atmosphere and then adapted and maintained in DMEM medium supplemented with 10% (v/v) FBS, glutamine 2mM and penicillin/streptomycin (0.01%/0.063%) at 37°C in a humidified 5% CO2 atmosphere.

In order to detach cells from the plate for subculturing, they were washed with PBS and incubated with trypsin for several minutes at 37ºC (0.5 ml of trypsin for 10 cm dish). Afterwards, cells were resuspended in 10 ml of fresh media. The appropriate passage of cells was performed, usually ranging between 1:4 and 1:12, depending on the cell type.

b) Freezing/thawing

Cells from a confluent 10 cm dish were collected and resuspended in 1 ml of ice-cold freezing medium (90:10 fetal bovine serum / DMSO or 90:10 normal medium / DMSO) and transferred to a 1.5 ml cryo-tube (Nunc). Cells were first stored at -20ºC 2hours, then at -80ºC for 2-3 days and finally were transferred to liquid N2 for long-term storage. Cells were quickly thawed by warming them in a 37ºC water-bath. Next, the suspension was transferred into a 10 cm dish with 15 ml of pre-warmed growth medium. The next day, the medium was replaced for a fresh one.

c) Generation of stable cell lines

GRK2-184B5 cells

Stably transfected 184B5 cells over-expressing GRK2-wild type were generated in 184B5 cells by using Fugene HD, selected with 200 ng/ml hygromycin and collected as pooled positive transfectants. A summary of the different positive clones generated is represented in fig. MM3.

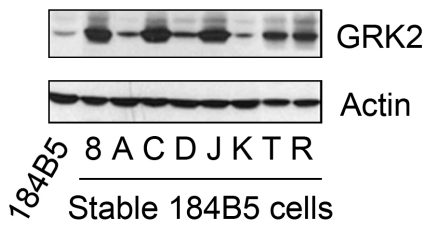


Figure MM.3: Generation of stable GRK2-over-expressing 184B5 cells. The pooled positive clones were lysed using RIPA buffer and immunoblotted with GRK2 to characterize the over-expression levels of GRK2. A representative western blot of the clones used in this thesis is represented.

GRK2wt / GRK2K220R MCF7-TET-ON system

MCF7-TET-ON system over-expressing GRK2-wt or the mutant GRK2-K220R was generated in our lab following the instructions of T-REXtm System (Invitrogen). The T-REXtm System is a tetracycline-regulated mammalian expression system that is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest (Yao et al., 1998). The mechanism of Tet-mediated repression is briefly summarized in fig. MM.4.

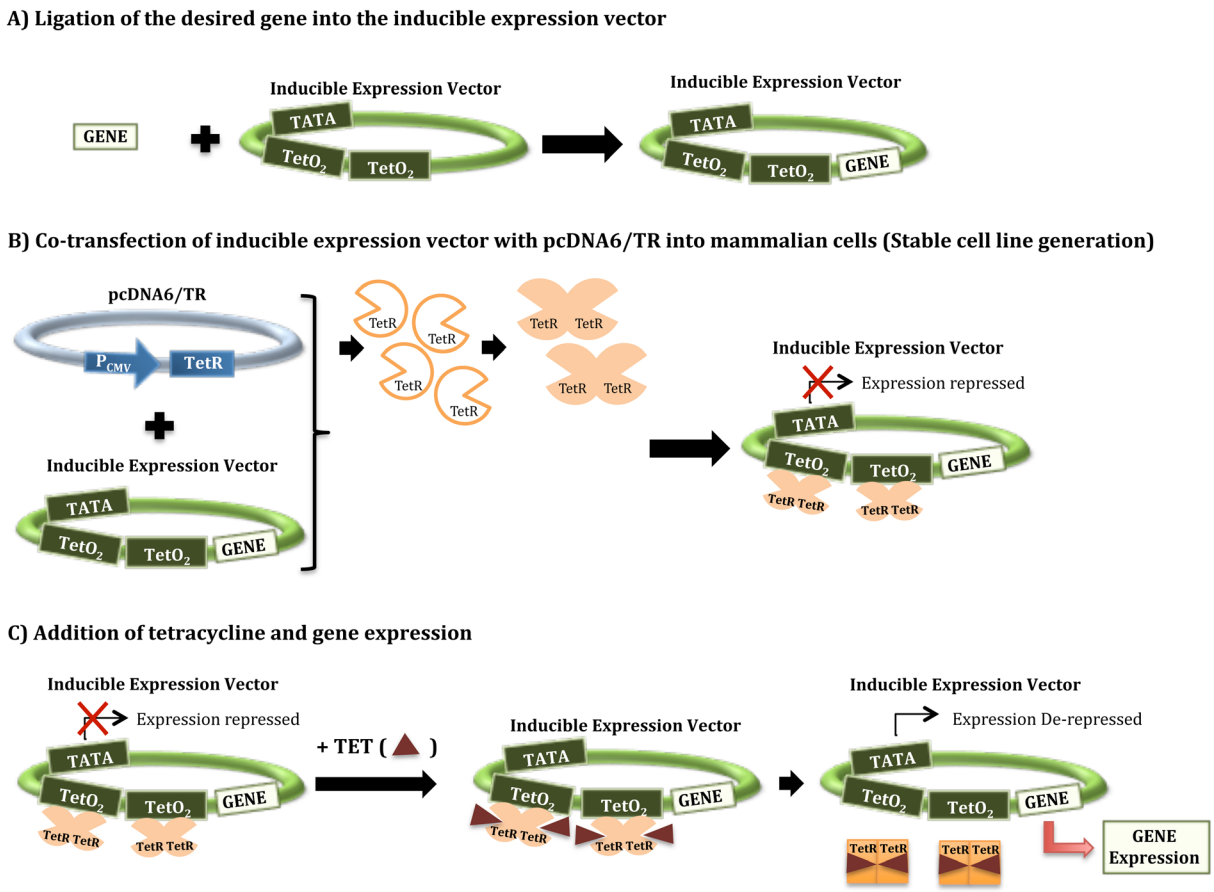


Figure MM.4: Tetracycline-controlled gen expression. A) Ligation of the desired gene into the inducible expression vector provided by Invitrogen. B) Cells were transfected according to the requirement of the chosen cell type. In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to each TetO2 sequence in the promoter of the inducible expression vector. Binding of the Tet repressor homodimers to the TetO2 sequences represses transcription of the gene of interest. C) Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest.

To start the process, the GRK2 wt or the kinase death mutant (GRK2-K220R) were cloned into the inducible expression vector, and the resulting construct co-transfected with the regulatory plasmid, pcDNA™6/TR into MCF7 mammalian cells using lipofectamine/ plus reagent. After 48 hours of transfection, cells were selected with blasticidin and Zeocin to isolate a single stable cell line expressing both the Tet represor and our gen of interest. Cells are treated with tetracycline (1 μg/ml) for several hours to check the inducible transcription of GRK2 or K220R (Fig. MM.5).

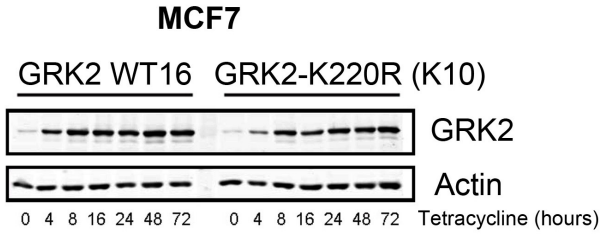


Figure MM.5: Inducible expression of GRK2 wild-type or K220R in MCF7 cells. The TET-On MCF7 cells with the repressed GRK2 wt or the kinase-dead mutant were treated with tetracycline (1 μg/ml) for the indicated times. Lysates of these cells were immunoblotted with GRK2 to check for protein expression induction.

HDAC6-GFP/ -HDAC6-S1060/62/68A-GFP MDA-MB-231 cells

MDA-MB-231 stable over-expressing HDAC6 and the GRK2-dependent triple phosphorylation defective mutant HDAC6-S1060/1062/1068A were generated using lentiviral constructs. First, the infectious lentivirus particles are generated using the Gateway system as described above and packed using the 293T cell line. We used lipofectamine2000 to reverse co - transfect 293T cells with three plasmids: (1) the lentiviral vector pDEST-DH1 that contains GFP-HDAC6 wild type, the triple mutant of phosphorylation sites (1 μg) or an empty pDest vector, (2) the pVSVG plasmid (1.5 μg) and (3) the psPAX2 plasmid (1.5 μg) with gag, pol, and rev genes, allowing virus to form the capside and to infect host cells. A suspension of 10^6 293T cells per condition was incubated for 18h at 37ºC and 5% CO2 with the transfection mixture diluted in OPTIMEN following the manufacturer's indications. Medium was replaced the next day by normal medium containing antibiotics and maintained for 24-48 hours. Virus-containing supernatants were collected and centrifuged at 5000 g for 2 minutes at room temperature to eliminate 293T remains.

Upon supplementation with polybrene (which aggregates virus particles) to a final concentration of 8 μg/ml virus-containing supernatants were added to the MDA-MB-231 cells (250000cells/M6 well). After 24-48h, medium was replaced with culture medium containing puromycin (10 ng/ml) for selection, and surviving colonies were collected and analyzed for western blot to check the expression levels of our proteins as shown in the fig. MM6.

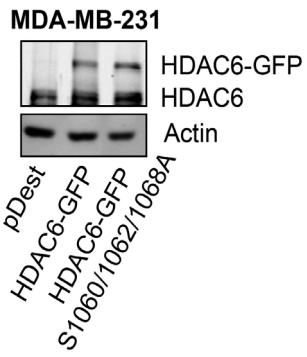


Figure MM.6: Generation of stable HDAC6-wt or S1060/1062/1068A GFP-over-expressing MDA-MB-231 cells. The pooled positive clones were lysed using RIPA buffer and immunoblotted with HDAC6 to characterize the over-expression levels of HDAC6.

d) Transient transfection-infection

Lipofectamine/Plus Method

Sub-confluent cells were transfected with the indicated combinations of cDNA or shRNA constructs by means of the Lipofectamine/Plus reagents (Invitrogen) diluted in OPTIMEN, following manufacturer's instructions. When indicated, cells were transiently co-transfected with a cDNA encoding the CD8 antigen for subsequent cell selection by using polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450, Dynal Biotech, Oslo, Norway). The transfection medium was replaced after 3h of incubation by growth medium to lower the cytotoxic effect of lipofectamine and cells were maintained 24 or 48 hours.

"Nucleofector" Cell Electroporation

184B5, MCF10A and MDA-MB-231 cells were transiently transfected using the Cell Line Nucleofector System (Lonza), following manufacturer's instructions. Cells cultured at 75-80% confluence were trypsinized and a pellet of 2*10^6 cells per condition was resuspended with a 100ml mixture of kit solution V plus 1-2mg of DNA or 20-400nM of siRNA. Quickly, this mixture was transferred into a certified cuvette provided by LONZA and electroporated using the program P-020 or X-013 for 184B5/MCF10A or MDA-MB-231 cells, respectively.

Lullaby™ siRNA Reverse Transfection

For transient siRNA transfections, cells were also reversely transfected with Lullaby™ (OZ-Biosciences), following manufacturer's instructions. Briefly, boht Lullaby reagent (8 mL/100 mL) and siRNA (optimal concentration of 25 nM) was diluted in culture medium without serum (OPTIMEM) The siRNA mixture was added into the diluted Lullaby reagent and mixed immediately and carefully 4-5 times by pipetting up and down. After 20 min. incubation at room temperature, the siRNA /Lullaby complexes were poured on cell culture dishes and a suspension of cells (2*10^6 cells per condition in 2 ml of culture medium containing serum) was added to the preformed complexes. Cells were cultivated under standard conditions for 24-48 hours after transfection, but sometimes assays were monitored up to 96 hours post-transfection.

Adenoviral infection

Cells are infected with adenoviral constructs for GRK2 (GRK2-wild-type, GRK2-K220R or shGRK2), with an adenovirus control or with a combination of shGRK2 and GRK2-K220R constructs (100MOIS) in OPTIMEM medium supplemented with 2% FBS for at least 6 hours. After 6-12 hours of infection, the infected medium was replaced with normal growth medium and cells were maintained for 24 or 48 hours.

e) Cell treatments

MDA-MB-468 cells cultured at 80% confluence in DMEM 10% FBS were treated with EGFR inhibitor AG1478 (500nM).

MCF7 and T47D cells cultured in phenol free DMEM-F12HAMS (GIBCO) at 80% confluence were oestrogen-starved for 48-72 hours (using Charcoal Serum) and then treated with 17b-estradiol (20nM) or Tamoxifen (1 or 5 μM).

To measure resistance to genotoxic agents, 184B5 or MCF7 cells were treated with doxorubicin (0,9nM), Paclitaxel (0.5μM), Etoposide (2μM) or cisplatin (0.5μM) for 0.5, 1, 2, 10 or 36 hours.

To inhibit different aspects of Mdm2 action over p53, MCF7 cells were treated with nutlin3a, an inhibitor of Mdm2-p53 interaction (1, 5 or 10 mM) or with the ubiquitinase activity inhibitor HLI737 (3μM)

184B5 and/or MDA-MB231 cells were serum-starved (0.1-1% HS or FBS) for 4 hours or ON and stimulated with Heregulin (20ng/ml), EGF (40 or 100ng/ml), SDF1a (50 or 100 ng/ml), CCL21 (60nM) or FBS (20%) .

184B5 and MDA-MB-231 cells were treated with HDAC6 inhibitors SAHA (3μM) or tubacin (10μM). To determine the IC50 of SAHA effects in MDA-MB-231 cells, cells were treated with different dosages of SAHA (0, 12.3nM, 30nM, 76.8nM, 192nM, 0.48μM, 1.2μM, 3 μM and 7,4 μM) and viability was analyzed by using xCellLigence System (Roche)

2.3 Animal models

MyrAKT transgenic mice were generated as described in (Blanco-Aparicio et al., 2007). Mammary glands from nine weeks old transgenic mice and wild type littermates were provided by Amancio Carnero's group (IBiS, Universidad de Sevilla, Spain). Paired non-tumoural and tumoural mammary glands samples from **transgenic FUB/N-tg (MMTVneu) mice** (Jackson) were provided by Santos Mañes's lab (CNB, Madrid). Snap-frozen mammary tissue was ground to a fine powder in liquid nitrogen using a mortar and was re-suspended in buffer A (50 mM TrisHCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP40 and 5% sodium deoxycholate) supplemented with a protease inhibitor cocktail (Leupeptin, Aprotinin, Orthovanadate, NaF and PMSF). Homogenates were centrifuged at 10000g for 10 min at 4ºC twice, and fat was removed from the surface. Final supernatants were analyzed by western blot.

6-8 weeks old **athymic nude-FOXN1 nu/nu** were provided form Harlan and kept in conditions of 22 ± 2 º C temperature, 45-55% humidity, rate of light / dark 12/12 hours and food and water "ad libitum".

2. 4 Preparation of cell lysates

a) Cell lysis

To prepare cellular lysates, collected cells were resuspended in 100-1000 µl of ice-cold RIPA lysis buffer with protease/phosphatase inhibitors, transferred to a precooled 1.5 ml reaction tube and incubated in a rocker (250rpm) for 1 hour. Afterwards, the lysates were centrifuged for 10-15 minutes at 4°C in a microfuge (14000 rpm). The cleared lysates were transferred to fresh pre-cooled 1.5 ml reaction tubes and stored at -80 °C.

b) Immunoprecipitation

RIPA lysis buffer was utilised for immunoprecipitations with Mdm2 antibody. To detect MDM2-P53 co-immunoprecipitation, a low detergent lysis buffer was preferred (see p53-Mdm2 interaction buffer in materials section). 5 ml of anti-Mdm2-agarose beads monoclonal antibody (0.5 µg/ µl) or anti-p53-agarose beads (0.5 µg/ µl) monoclonal antibody were incubated with 1 mg of cell lysates in a rocker (250rpm) over-night at 4ºC. The beads were then washed 5-7 times in RIPA or p53-Mdm2 interaction buffer and analyzed by immunoblotting.

c) Subcellular fractionation

Cells were washed with PBS and collected in hypotonic buffer (200 µl/10 cm dish), and incubated in ice for 10 min. After addition of 1% of NP40 cells were centrifuged at 3.000 rpm for 5 min a 4 ºC. The supernatant was frozen and thawed in dry ice three times and cleared by centrifugation at 10000 rpm for 10-15 min at 4ºC. This fraction was taken as the cytosolic fraction and kept at -80ºC. In turn, the initial pellet was washed twice by centrifugation with hypotonic buffer supplemented with 1% NP-40 and lysed in 100 µl of buffer C for 20 min on ice. Finally, buffer C lysates were centrifuged 10 min at 10000 rpm at 4ºC. The supernatant was taken as the nuclear fraction. For immnubloting, we usually used 10 µg of nuclear fraction and 30 µg of cytosolic fraction.

d) Nuclear extraction kit

Nuclear extracts from adherent cells were prepared using the Nuclear Extraction Kit (Panomics) according to the manufacturer's instructions.

e) Determination of protein concentration

Protein concentration in cellular lysates was determined using the DC Protein Assay Reagents A, B and S (Bio-Rad). 3 µl of cellular lysates were mixed with 25 µl of solution

obtained by the combination of 10 μl DC Protein Assay Reagents S and 500 μl of Reagent A. Afterwards, 200 μl of Reagent B was added and the solution was incubated 10 minutes at room temperature. Absorbance at 750 nm was measured on a Biorad iMark microplate Reader. Protein concentration was determined with respect to the absorbances of known concentrations of BSA protein. In general, 15-20 μg of total protein were used for western blotting.

2. 5 Immunoblotting

Cellular lysates/immunoprecipitations were separated by SDS-PAGE on a 6-15% Acrylamide/N,N'methylen-bisacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes (0.45 mm, BioRad) using a wet blotting apparatus (Bio-Rad). For metabolic labeling and pulse-chase experiments, proteins were transferred to previous methanol-activated-PVDF membranes (Millipore). The efficiency of transference was always evaluated by staining with Ponceau staining solution. Ponceau was washed out with TBS-tween and the membranes were blocked for 1 hour in TBS-tween supplemented with 5% BSA at room temperature. Membranes were incubated with the primary antibodies primary antibodies in TBS-Tween supplemented with 3% BSA overnight at 4°C (see Materials for antibodies dilution). Afterwards, the blots were washed three times for 10 minutes in TBS-Tween followed by incubation with the secondary antibodies 1 hour at room temperature (see Materials for antibodies dilution). After extensive washing with TBS-Tween, membrane-bound secondary antibodies were detected using ECL (Enhanced ChemiLuminiscence) from Amersham Pharmacia Biotech and Agfa films. Alternatively, in the case of fluorescent secondary antibodies, the Odyssey Infrared Imaging System was utilised. Bands were quantified by laser densitometry with a Biorad GS-700 scanner or by the software included in the Odyssey Infrared Imaging System.

Staining gels with Coomassie Blue

In some cases, proteins were directly visualized with a coomasie staining solution (See materials). After SDS-PAGE electrophoresis, gels were stained for 20 min with coomasie and then destained with coomasie destaining solution until proteins became visible (1-2 h usually). Afterwards, gels were washed with distilled H2O for 20-30 min and dried in a gel dryer at 80°C for 45 min.

2.6 Protein/DNA arrays

The activity of transcription factors (TF) was examined by using a protein/DNA Arrays from Panomics. The procedure is simple and straightforward and involves three

steps: The first one requires a pre-incubation of biotin-labeled DNA binding oligonucleotides with a nuclear extract of interest (obtained following instructions of Nuclear Extraction Kit from Panomics) to allow the formation of protein/DNA complexes. In the second step, proteins/DNA complexes were separated from free probes, and the last step is the hybridation with the protein/DNA Array. Finally, the array was detected with a HRP-based chemiluminiscence detection method.

2.7 Protein Stability Assays

a) Cycloheximide treatment

Protein decay of doxorubicin-induced p53 was determined using translation shut-off assays. 184B5 cells with (184B5-A) or without extra GRK2-wt were seeded at 80% confluence in a M12 well plate and incubated for 2h with the DNA-damaging agent doxorubicin (0.9μM) prior to the addition of 20μg/ml cycloheximide or vehicle, that was maintained during the chase periods to inhibit protein synthesis.

b) Pulse-chase assay

Metabolic labeling and pulse-chase experiments were performed as described (Penela et al., 2001). HEK-293 cells transfected with Mdm2 in the presence or absence of wild-type GRK2 or GRK2-K220R were pulse-labeled for 15 min. Upon Mdm2 immunoprecipitation, protein complexes were resolved by SDS-PAGE and transferred to PVDF membranes (previously treated with methanol for 5 min) to be developed using the Amersham hyperfilm from GE Healthcare Live Sciences. Band density of 35S-labeled Mdm2 was quantitated by laser densitometry analysis and data were corrected according to total Mdm2 protein detected by immunoblotting.

2. 8 Determination of the phosphorylation sites of Mdm2 by GRK2

a) Protein kinase assay

Recombinant GRK2 protein (50nM) was incubated with or without the indicated purified GST-Mdm2 constructs (100nM) or GST in 50μl of kinase assay buffer. For the analysis of the GRK2-mediated phosphorylation of different Flag-Mdm2 mutants, recombinant GRK2-wt (100nM) were mixed in kinase assay buffer with immunoprecipitated tagged full-length or mutant constructs of Mdm2 expressed in cells. After 30 minutes at 30ºC, the reaction was stopped by the addition of SDS-sample buffer. Phosphorylated proteins were resolved by 6-10 % SDS-PAGE, stained with Coomassie and visualized by autoradiography for 4 to 24 h.

b) Proteomic approach for the identification of phosphorylated peptides

GST-Mdm2 fusion protein was subjected to a protein kinase assay with recombinant GRK2 protein in the presence or absence of cold ATP (the procedure is the same as detailed in section above except for the absence of radioactive ATP) and then analyzed by mass spectrometry to identify possible phosphorylation sites in Mdm2 by GRK2. First, non-phosphorylated Mdm2 was digested by tripsin and chymotrypsin to obtein the sequence coverage and then candidate peptides to be phosphorylated by GRK2 were analyzed by LC-MS/MS. The proteomic "Protein identification by LC-MS/MS (Ion Trap)" analysis was carried out in the 'CBMSO PROTEIN CHEMISTRY FACILITY', a member of ProteoRed network.

2.9 Ras activity assay

Cells were serum starved for 4 hours and treated wit EGF (100ng/ml for 2,5, or 20 min). Cells were lysed in RIPA buffer and 25 μg of cell lysates were subjected to the G-lisa assay (Cytoskeleton) to measure Ras activation following manufacturer's instructions. Absorbance was read at 490nm. Data are background subtracted.

2. 10 Cell proliferation Assays

a) MTT

The measurement of cell viability in MCF7 and MDA-MB-468 cells was performed by tetrazolium salt-based colorimetric assays (MTT) that account for the number of active mitochondria in a cell population. 5000 cells were added to the wells of a 96-well plate in DMEM with 10%FBS. Each 24 hours, 20μl/well of CellTiter 96(R) AQueousOne Solution Reagent (Promega) was added. After 1 hour at 37ºC in a humidified, 5% CO2 atmosphere, the absorbance at 490nm was recorded using an ELISA plate reader. The background absorbance shown at zero cells/well was subtracted from the data.

b) Real-time monitoring of cell adhesion, proliferation and cell viability using the xCELLigence System (Roche Applied Science).

The conceptual basis of this technology is that cells when attached to the plate act as mini-condensers and change the electrical impedance that is being continuously recorded by means of several electrodes located underneath the plate. (Fig. MM.7A) Cellular impedance was converted to a cell index (CI) that allows for the assessment of different cellular processes. As cells attach and proliferate over time, impedance rises as does the cell index recorded. Changes in their adherence, viability and morphology also change

the impedance and can thus be monitored. Thus, cell proliferation, cell surface coverage, cellular adhesion strength or cell viability can be measured in a real-time label-free cellular setting. This technique has been previously shown to strongly correlate with classical methods for the detection of these cellular processes (Limame et al., 2012).

For each experiment growth media baseline impedance was measured in each well before the addition of the cells to ensure all changes recorded were normalised to those initial values determined by the ionic composition of the media. Approximately 5000 cells were added per well onto a E-Plate 16 and monitoring started shortly afterwards. Continuous impedance measurements were monitored every 15 minutes for varying times (70-150hrs). In low-serum proliferation assays, an initial amount of 10000 cells were seeded per well.

In order to define the specific parameters to be measured in each experiment a careful analysis of the global kinetics is necessary. The first 8-16 h after the cells were plated correspond to the adhesion phase (see fig. MM.7), that represents the necessary time to complete total spreading of the cell. Usually within 4-6h the cells were already attached, therefore cell index values were normalized at 4-6 hours to analyze proliferation events. Cell growth was calculated as the slope (hours-1) of the cell index curve using the RTCA software integrated in xCELLLigence system.

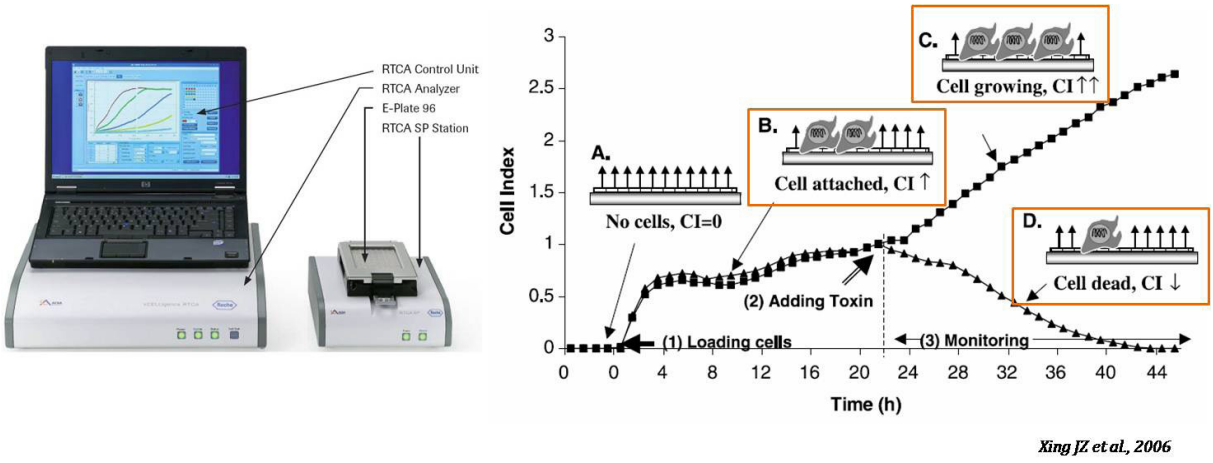


Figure MM.7: Real-time monitoring of cell behavior using the XCellLigence System. Roche Applied Science has developed a system by which electrical impedance is continuously recorded by means of several electrodes located underneath the plate that is directly connected to a computer. Once cells start attaching to the plate, cell index increases its value. Cell adhesion (B) and morphological changes were assessed in the first 16-20 hours. After that, proliferative events were recorded with a detection of slope change. (C). To analyze the effects of cytotoxic agents, the toxin was usually added once cells have been attached to the plate. Negative slopes show cells detaching from the plate due to cell death.

Drugs treatment- real time monitoring and IC50

Cell proliferation assays using several cancer cell lines were carry out to assess the efficacy and potency of different anti-cancer compounds. The xCELLigence system was used to quantitatively and dynamically monitor cell cytotoxicity. After addition of a cytotoxic agent, cells start to die changing their morphology and detaching from the plate, what results in a decrease in cell index value and in a negative growth slope (fig MM7.Bd). CELLigence system allows the assessment of important parameters such as rate and onset of cytotoxicity as well as calculation of time-dependent IC50s.

To determine saha and tubacin effects, cell growth was sorted out in two different time frames. The first lapse ranges from 0 to 40 hours to analyze proliferative events, and the second lapse goes from 40 to 150 hours to determine the effects of treatment when non-treated control cells start to die. Cell index values were normalized at 4-6 or at 40 hours respectively and growth slope was calculated as indicated before. To calculate IC50, different dosages of SAHA (from 0 to 7.4 μM) were added to MDA-MB-231 cells seeded by duplicate or triplicate and IC50 values were calculated as the area unser the curve using the software integrated in XCellLigence system (sigmoidal response-slope variable). In a similar way, the effects of nutlin3a (1, 5 and 10 μM) were also analyzed by XCellLigence in MCF7 cells.

2. 11 Colony formation in Soft Agar - Anchorage independent growth assay

5000 Cells were re-suspended in DMEM containing 0.3% low-melting agarose and 10% FBS (upper layer), and seeded onto a coating of 0.7% low-melting agarose in DMEM containing 10% FBS (Bottom layer). Once a week an extra upper layer was added to allow for the formation of colonies and growth. (Fig. MM.8)

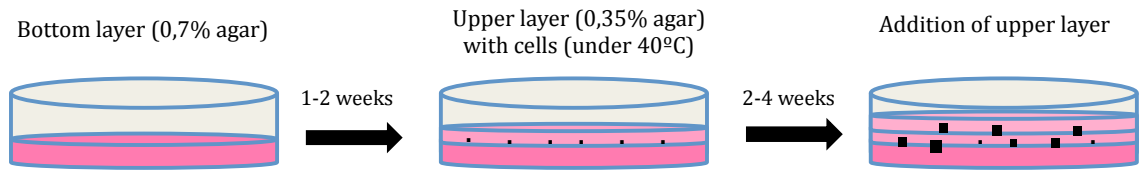


Figure MM.8: Colony formation in Soft Agar scheme. The soft agar colony formation assay is a common method to monitor anchorage-independent growth, which measures proliferation in a semisolid culture media after 3-4 weeks. Agar plates were prepared in 35-mm petri dishes by first applying 1.5-ml bottom layer of 0.7% agar in growth medium. Over this basal layer, an additional ml layer of 0.3% agar in the same medium with the appropriate concentration of cells was added. Cells were incubated at 37ºC in a humidified atmosphere of 5% CO2 in air and refed after 7 days by addition of 1ml of 0.3%agar/ growth medium. Colonies were measured, fixed and stained with crystal violet. Agar growth assay could be scored at 1 and 3 weeks.

After 1 or 3 weeks of growth cells were fixed with methanol and stained with 0.005% Crystal Violet. Number and size of colonies were scored. Z-stacks were acquired using a a 10X objective of a Zeiss microscope with a Coolsnap FX color camera. The area of 100-200 colonies per each condition was measured with Image J. Colonies were distributed in three groups according to their size: Large, Medium and Small, which were defined by the ratio between the median area of each group and the median area of the whole colonies in each of two independent experiments performed in duplicate (Ratio for large colonies = 5; Ratio for medium colonies = 2; Ratio for small colonies < 1). The number of colonies in each group was expressed as the percentage over the total colonies. Average Size (area) of large colonies were also scored and expressed as the fold-increase over the control condition.

2. 12 In vivo Tumour Implantation and immunohistochemical assays.

MCF7cells (1 x 10^6), MDA-MB-231 cells (5-10 x 10^6) or MDA-MB-468 cells (10 x 10^6) in 100µl of PBS supplemented with 0.1% glucose and containing Matrigel (3,3mg/ml) were subcutaneously implanted on the back of 8-10-weeks-old male and female atymic nude mice (Atymic nude-Foxn1 nu/nu)(6-8 mice/group). Tumour growth was monitored once every three days with callipers. Tumour size was calculated as volume of tumour (mm^3) with the formula [length (mm)2 x width(mm)] x 0.5236. When tumour volume exceeded the 10% of the total weight of the animal (between 1500-2,000 mm^3), mice were sacrificed for ethical reasons. Tumours were processed for histological analysis to analyze the expression of different proteins. MCF7 Tet-on inducible cells were injected in mice pre-treated for 24 with doxycicline (Clontech, 2 mg/ml supplemented with 5% sucrose in drinking water). Once a week, doxycicline was replaced in the drinking water. Vehicle-treated MCF7-TET-ON cells were injected in non-treated mice as a control.

After 8 or 30-35 days post-implantation, tumour mass was excised and fixed with 3.7% paraformaldehyde (PFA)/PBS. Paraffin-embedded serial sections (3µm thickness) were de-waxed with xylene and rehydrated, followed by blocking of non-specific binding and endogenous peroxidase activity 30 minutes with 5% normal horse serum and 20 min with 3% H2O2 solution, respectively. Slides were stained with specific antibodies α-rabbitGRK2 (1:1000), α-rabbitKi67 (1:300), α-mouse p-53 (1:400), α-rabbit-cleaved-caspase3 (1:200) antibodies. Sections were then overlaid with secondary biotinylated goat anti-mouse or rabbit IgG, followed by incubation with streptavidin-peroxidase conjugate (ABC Elite Kit Vector) to amplify the signal. For chromogenic localization of antibodies, 3,3'-diaminobenzidine (DAB) was used. After optimal colour development, sections were

counterstained with Gill 2hematoxylin (Thermo Scientific). Protein immunostaining was quantified in 15-40 fields covering the entire tumour and represented as the area of positive cells over the total area of cells. Data were obtained from 15-40 images taken from tumours of at least 2 different mice.

2.13 Immunohistochemistry Analysis of Human Breast Tumours.

For immunohistocheminal analysis of human breast samples, a tissue microarray of 5-μm core sections from 49 metastatic infiltrating ductal carcinoma samples (US Biomax) was used. The sections were deparaffinised and rehydrated in water, after which antigen retrieval was carried out by incubation in EDTA solution, pH 8.2 at 50ºC for 45 minutes. Endogenous peroxidase and non-specific antibody reactivity was blocked with peroxidase blocking reagent (Dako) at room temperature for 15 minutes. The sections were then incubated for 60-90 minutes at 4ºC with the anti-GRK2 PF2polyclonal antibody and with phospho-S473-AKT polyclonal antibody. Detection was performed with Envision Plus Detection System (Dako). Negative controls were used with goat serum replacing the primary antibody. The slides were counterstained with haematoxylin and after drying were mounted with DPX mountant for microscopy (VWR Int).

2.14 Immunofluorescence and confocal microscopy

Cells were rinsed in PBS, fixed in 4% paraformaldehyde (PFA) for 30 min and washed again with PBS. When indicated, cells were treated with 4% PFA plus 0.5% triton for 1-2minutes (before fixation with PFA alone) or with 0.5% triton in PBS for 10 minutes (After PFA fixation). Non-specific sites were blocked by incubation in PBS containing 1% BSA and 0.5% Triton-X100 for 1 h at RT. Cells were then washed 4 times in PBS and incubated 1 h with anti-cortactin monoclonal antibody (1:200). After four washes with PBS, cells were incubated 1 h with Falloidin-FITC and Alexa Fluor 650 secondary antibody (1:200, Molecular Probes), washed again with PBS and incubated for 5-10 min with DAPI (1:5000). Finaly, samples were mounted in mowiol or prolong dapi (with this mounting medium the previous incubation with DAPI is not necessary). Samples were examined by confocal microscopy (Zeiss, Germany) or a widefield microscope DM6000 B/M (Leica Microsystems) equipped with a CCD CoolSnap HQ camera (Roper Scientific). The quantitative analysis of fluorescence intensities was performed using a confocal laser microscope and the Analysis of Image System (LSM 510, Zeiss, Germany), Image J (http://rsb.info.nih.gov/ij/) or Metamorph (Molecular Devices Corp., Sunnyvale, CA)

2.15 Cell Migration assays

a) Conventional Transwell for detection of cell migration

A Transwell setup consists of an upper chamber (insert) that is placed onto a lower chamber (well). The insert contains a microporous membrane (6.5-mm Transwell filters with 8-μm pores (Costar) allowing passage of tumour cells. After a period of serum starvation (1% FBS during 18h hours or 0% FBS during 4 hours) a serum-free cell suspension (40000 cells) is seeded in the insert and exposed to medium containing potential chemoattractants. During 5 hours of incubation at 37ºC and 5% CO2, cells migrate toward the bottom side of the membrane. The experimental design to assess time-dependent migratory behavior of cultured cells is represented in fig. MM.9A. Both migration toward quemoattractant-containing medium and baseline migration (toward starving medium, no chemoattraction) as a negative control were included. Fibronectin (20 μg/ml)-coated filters were used to potentiate adhesion of MDA-MB-231 cells. After 5 hours of incubation, cells were fixed with ice-cold methanol, stained with DAPI 5μg/ml and counted in five random fields of each filter.

b) Real time Cell migration by using xCellLigence

Cell migration experiments were performed using modified 16-well plates (CIM-16 plate, Roche Diagnostics, Spain) with each well consisting of an upper and a lower chamber separated by a microporous membrane containing randomly distributed 8 mm-pores. This setup corresponds to conventional Transwell plates with microelectrodes attached to the underside of the membrane for impedance-based detection of migrated cells (Fig. MM.9B). Prior to each experiment, cells were deprived of FBS ON. Initially, chemoattractants (140mL) or serum-free media (30mL) were added to the lower and upper chambers respectively. The CIM- 16 plate was locked in the RTCA DP device at 37ºC and 5% CO2 and a measurement step was performed as a background signal generated by cell-free media. To initiate an experiment, cells were detached, resuspended in serum-free (SF) medium, counted and seeded in the upper chamber applying 40000 cells in 100 mL. After cell addition, continuous impedance measurements were monitored every 15 minutes for 24 hours. Both directed migration towards 10% FBS or chemoattractant compounds (Chemotaxis) and random migration supported by SF medium on either side of the membrane (Chemokinesis) have been considered. All raw data obtained were normalized to the single maximal value (CI) to eliminate units of measurement. Subsequently, signals representing net chemoattraction were obtained by subtracting background (SF) values from the positive control (medium containing FBS) signals as described in (Limame et al., 2012).

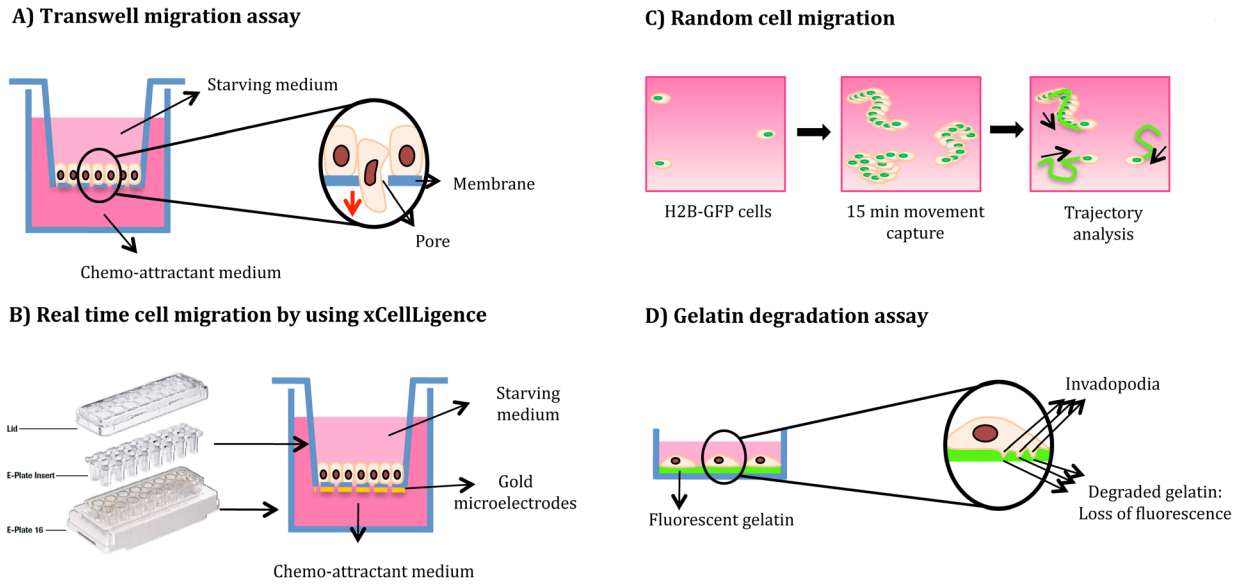


Figure MM.9: Schemes of commonly used 2D-migration and invasion assays. An overview of the technical setup is schematically drawn for each assay and a close-up view is given right to it (inside the big circles). Arrows indicate the direction of cell movement. A) Transwell migration assay. Migration of a cell through a pore in the membrane is depicted. B) Real time cell migration (xCellLigence). Migration of a cell through a pore in the membrane takes place in the same way as in section A, but this system incorporates gold microelectrodes that sense impedance upon contact of cells crossing the pore. C) Random cell migration. H2B-GFP Cells were seeded onto a collagen type I bottom layer and monitored each 15 minutes. Cells perform radial movement, the area of which can be measured by projecting all the movements of the same cell in 72 hours. (D) Gelatin degradation assay. Cells are seeded onto a thin fluorescently labeled gelatin layer. At sites of invadopodia mediated matrix degradation a loss of fluorescence occurs, which can be documented and quantified by microscopic imaging.

c) Random cell migration in 2D onto a collagen type I layer

To analyze the motility and invasion of H2B-EGFP MDA-MB-231 tumour cells in sparse culture conditions on two- (2D) collagen I matrix, H2B-EGFP expressing MDA-MB-231 cells were reverse transfected with Lullaby reagent (OZ Bioscience, France) plus 25 nM MT1-MMP or GRK2 siRNAs ON-TARGET PLUS SMARTpool PLUS (Dharmacon). 24 hours after transfection, 2000 cells/well were seeded in a 96 well-plate coated with a thick bottom layer (≥100 mm) of acidic-extracted type I collagen. After polymerization, L15 medium containing 15% FCS and 2 mM l-glutamine was added to each well. H2B-EGFP time-lapse sequences were recorded at 15-min intervals for 48 h over random fields (see fig. MM9.C). For time-lapse microscopy, we used a Nikon TE2000 microscope equipped with a 10× NA 0.75 objective controlled by the Metamorph software (Molecular devices). This microscope was equipped with a cooled CCD camera (HQ2, Photometrics). To quantify cell motility, we determined a "displacement index" (DI) by

dividing the area over which the H2B-EGFP-positive nuclei moved in a 48-72 hours time lapse (as result of the time lapse projection of the frames recorded every 15 min) by the area occupied by cells in the first frame of the time sequence.

2.16 Gelatin degradation assay.

MDA-MB-231 cells were transfected with 20nM of required siRNAs using the Cell Line Nucleofector System (Lonza). 48-72 h post-transfection, cells were trypsinized, counted and 30,000 cells/well were incubated for 5 h on FITC or Alexa Fluor 488-conjugated cross linked gelatin (Molecular Probes, Invitrogen) and then fixed and stained for F-actin and cortactin as described Rey et al, 2011 and Fig. MM9.D. Briefly, coverslips (18-mm diameter) were coated with 0.5 mg/ml poly-l-lysine (Sigma–Aldrich) for 20 min at room temperature, washed with PBS and fixed with 0.5% glutaraldehyde (Sigma–Aldrich) for 15 min. After three washes, coverslips were inverted on an 80ml drop of 0.2% fluorescently-labeled gelatin in 2% sucrose - PBS, and incubated for 10 min at room temperature. After washing with PBS, coverslips were incubated in 5mg/ml sodium borohydride (Sigma–Aldrich) for 3min, washed three times in PBS and finally incubated in 2ml of complete L15 medium before adding the cells. To measure gelatin fluorescence intensities, at least 100 cells from two different coverslips were analyzed in each experiment and positioned using a 63x immersion oil objective of a widefield microscope DM6000 B/M (Leica Microsystems) equipped with a CCD CoolSnap HQ camera (Roper Scientific) and steered by Metamorph (Molecular Devices Corp., Sunnyvale, CA). For quantification of degradation, two parameters of interest were addressed: The percentage of cells degrading gelatin over the total number of cells and the degradation index (DI), referred to the degradation intensity and calculated by the total area of degraded matrix in one field (black pixels) divided by the total number of phalloidin-labeled cells in the field. The degradation index of Mock-treated cells (i.e. transfected with non-targetin siRNA) was set to 100, and degradation indexes of all cell populations were normalized to this value and expressed as a percentage.

2.17 3D Cell Invasion assays

a) Matrigel-coated Transwell for detection of cell invasion

A protocol identical to the above transwell migration experiments (section 2.16.a) was followed for invasion experiments except for the application of a layer of Matrigel on the upper side of the membranes and dynamic process follow-up during 24 hours. Aliquoted Matrigel (Basement Membrane Matrix, growth factor reduced, BD Biosciences, Erembodegem, Belgium) was thawed overnight on ice and then mixed with ice-cold SF medium to obtain a final concentration of 250 mg/mL. Afterwards, 100 ml were seeded

onto each upper chamber of the transwell and let them dry ON at room temperature under sterile conditions. The next day, the matrix was rehydrated with SF medium and cells (100000) was added into the upper chamber and incubated for 24 hours at 37°C and 5% CO2 atmosphere. A scheme of the procedure was detailed in fig. MM.10A. To stop the experiments, cells were fixed with cells were fixed with ice-cold methanol and stained with DAPI 5µg/ml and counted in five random fields of each filter.

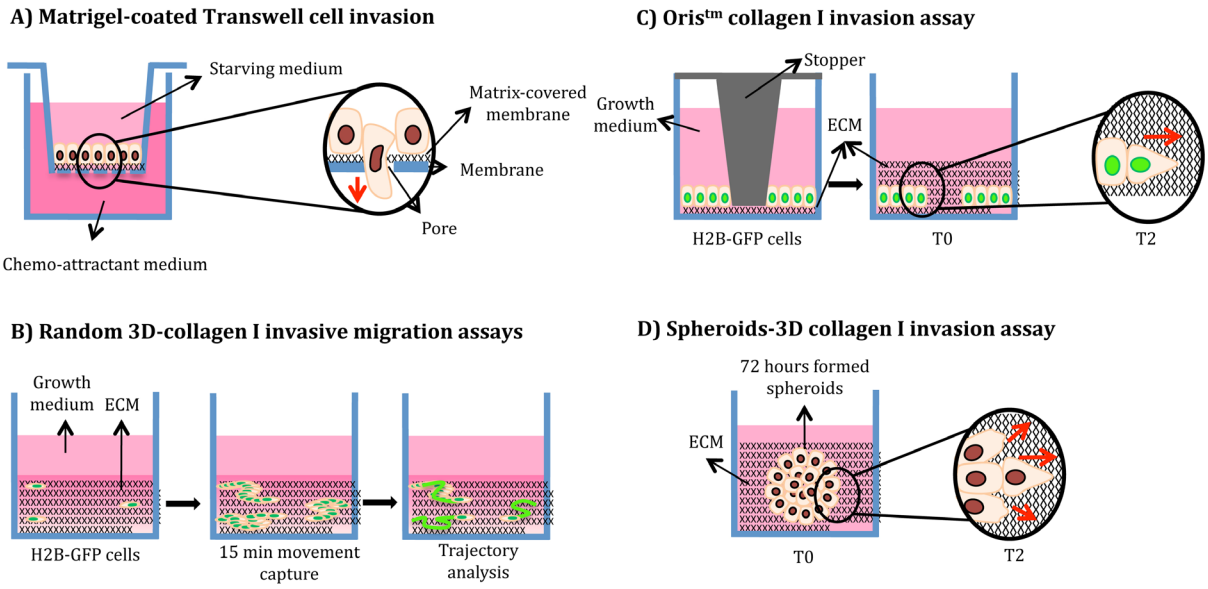


Figure MM.10: Schemes of commonly used 3D-invasion assays. An overview of the technical setup is schematically drawn for each assay and a close up view is given right to it (inside the circles). Red arrows indicate the direction of cell movement. Hatched areas symbolize ECM. A) Transwell invasion assay. B) Random 3D-collagen I invasion assay. H2B-GFP cells are tracked on their route through the ECM via automated fluorescent microscopy. Their routes through 3D space can be recorded and measured by following GFP nuclei. C) Oris™ Collagen I invasion assay. Cells are seeded on top of a thin ECM coated surface and are overlaid by a second thicker layer of ECM. In the center, a silicone plug creates a cell-free exclusion zone. Therefore, invasive cells are embedded in ECM and migrate from an outer ring into the center. D) Spheroid 3D-collagen I invasion assay. Multicellular spheroids are embedded into ECM gels, which are submerged in growth medium. Invasive cells emerge from the cell clusters and lead to astral outgrowing structures.

b) Random 3D-collagen I invasive migration assays

To analyze the motility and invasion of H2B-EGFP MDA-MB-231 tumour cells in sparse culture conditions in three-dimensional (3D) collagen I matrix, H2B-EGFP expressing MDA-MB-231 cells were reverse transfected with Lullaby reagent (OZ Bioscience, France) as in section 2.16.c and 24hours after transfection, 2000 cells/well were seeded in a 96 well-plate coated with a thick bottom layer (≥ 100µm) of acidic-extracted type I collagen. For assessing invasion in 3D collagen-I, after attachment, cells were overlaid with a 2.2 mg/ml type I collagen. After polymerization, L15 medium containing 15% FCS and 2 mM l-glutamine was added to each well. H2B-EGFP time-lapse sequences were recorded at

15-min intervals for 48-72 h over random fields (fig MM.10B). For time-lapse microscopy, we used a Nikon TE2000 microscope equipped with a 10× NA 0.75 objective controlled by the Metamorph software (Molecular devices). This microscope was equipped with a cooled CCD camera (HQ2, Photometrics). Cell movement was quantified using the displacement index factor (DI) of H2B-EGFP-positive nuclei over the time sequence.

c) Oristm collagen I invasion assay

H2B-EGFPexpressing MDA-MB-231 cells were reverse transfected with Lullaby reagent (OZ Bioscience, France) with 25nM of required siRNAs. 24 h post-transfection, cells were trypsinized, counted and 40,000 cells/well were seeded in the presence of OrisTM Cell Seeding Stoppers to restrict seeding to the outer annular regions of the wells in a 96 well-plate coated with a type I collagen bottom layer (Fig. MM.10C). Removal of the stoppers reveals a 2 mm diameter unseeded region in the centre of each well, i.e. the detection zone, into which the seeded cells may then invade for a 48-72 h time period, once a collagen I overlay has been applied. Type I collagen was prepared from acid extracts of rat tail tendon at a final concentration of 2.2 mg/ml (Elsdale & Bard, 1972). H2B-EGFP images were acquired from each well at the beginning (T0) and 48 h post-invasion (T2) using a Nikon TE2000 microscope equipped with an 4X objective CFI Plan Fluor NA 0.13 WD 17.1 controlled by the Metamorph software (Molecular devices). This microscope was equipped with a cooled CCD camera (HQ2, Photometrics). Image analysis was performed using Metamorph software. The index of invasion was determined by thresholding the area occupied by H2B-EGFP nuclei in the detection zone of each well after 48 h of invasion. This index is defined by the total area occupied by H2B-GFP nuclei in unseeded area (detection zone), at the end of the assay (T2) by subtracting the area occupied by H2B-GFP nuclei at the beginning of the experiment (T0). This area of invasion was normalized and results were presented as the fold increase of invasion over the control condition.

d) Spheroids formation and 3D collagen I invasion assay

Multicellular spheroids of MDA-MB-231 cells were prepared with the hanging droplet method (Kelm et al., 2003), using 3×103 cells in 20ml droplet in complete L15 or DMEM media. For siRNA treatment, cells were transfected by nucleofection with 200nM SMARTpool siRNAs specific for GRK2 or MT1-MMP. After nucleofection, cells were seeded in a Petri dish and spheroids were made for 72 hours. After 3 days, spheroids were embedded in type I collagen (prepared from acid extracts of rat tail tendon at a final concentration of 2.2 mg/ml (Elsdale & Bard, 1972)). Spheroids were fixed in 4% paraformaldehyde immediately after polymerization of the matrix (T0) or after 2 days of invasion (T2) (see fig MM.10D). After fixation, cells in spheroids were permeabilized for

15 min in 0.1% Triton X-100/PBS and labeled with Alexa545-phalloidin and DAPI. For quantification of invasion in 3D type I collagen matrix, phalloidin-labeled spheroids were imaged with a confocal microscope using a dry 5X CFI Plan objective, collecting a stack of images along the z-axis with a 10-15mm interval between optical sections. Spheroid-mean diameter was measured using ImageJ software macro language. The mean diameter was measured from azimuthal averaging of intensity profile along a line centered on the spheroid. This averaging consists in measuring intensity profiles along a rotating line by 5º steps and calculating the mean value over all angles of each pixel of the line. The mean diameter was then taken as the width at 1/10 of the maximal value of these averaged intensity profiles. Mean area (pr2) was calculated from the mean diameter. To rule out a proliferative component, spheroids were also stained with KI67 and imaged using a 10X objective to analyze the percentage of KI67 nuclei in the invasive areas. T2 data were normalized with their corresponding T0, and statistical analyses were performed between the different normalized-T2conditions.

2. 18 Statistics.

Data in all figures are expressed as mean ± SEM or SD as indicated. All results were confirmed in at least 2 separate experiments. Data were analyzed using Student's T-test. Two-tailed $p< 0.05$ was considered statistically significant.(*$p< 0.05$, **$p< 0.01$, ***$p< 0.001$). Statistical correlations obtained from samples of patients were analyzed using Pearson Test. (*$p< 0.05$, **$p< 0.01$, ***$p< 0.001$) and statistical analysis from spheroids 3D collagen I invasion assay was performed using Abnova two factor in GraphPad Prism 5 software.

2. 19 Study approval.

The different transgenic mouse models used in this study were housed and bred following all established regulatory standards, and all the experiments were performed in accordance with guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Directive 86/609/EEC) and with the authorization of the Bioethical Committee of the Universidad Autónoma de Madrid. Breast cancer samples from patients recruited at the Hospital Universitario La Paz (Madrid, Spain) were used for immunochemistry studies after approval by the Ethics Committee for Clinical Research of this institution. At the time of surgical patients were informed and provided consent that the excess of the rejected sample could be used for research purposes after diagnosis completion. A specific written consent from patients to participate in this particular study was not necessary since no additional sample was collected.

RESULTS

1.

GRK2 protein levels are enhanced in human transformed breast cancer cells of luminal epithelial origin.

Our group had previously described that sustained activation of the insulin-like growth factor-1 receptor (IGF-1R) promoted activation of AKT, which in turn phosphorylated Mdm2 and mediated its nuclear translocation (Salcedo et al., 2006). These events resulted in the inhibition of Mdm2-mediated GRK2 degradation, leading to enhanced GRK2 stability and increased kinase levels in both normal and tumoural mammary cell lines. Moreover, a constitutively active mutant of Akt (Akt-myr) markedly protected GRK2 from degradation, while pharmacological inhibition of Akt downmodulated the expression of GRK2 in different tumour cells (Salcedo et al., 2006). Interestingly, a significant proportion of all subtypes of human breast tumours displays increased activity of the PI3K/AKT signalling axis by means of mutations in PIK3CA or AKT genes or increased activity of the PI3K/AKT pathway by means of non-mutational alterations in signal transduction (Cancer and Atlas, 2012). In this context, it was tempting to suggest that deregulated AKT activity, by relieving the Mdm2-dependent degradation of GRK2, could stabilize/enhance kinase levels in some tumoural situations. Therefore,

we decided to examine the expression of GRK2 in a panel of breast tumour cell lines that mirrors many of the genomic abnormalities and molecular signatures found among the different subtypes of primary breast tumors (revised in (Vargo-Gogola & Rosen, 2007)). A comparative analysis of GRK2 protein levels showed that MDA-MB361, T47D and MCF7 cells, which derive from luminal breast cancers, expressed 3- to 10 fold higher kinase levels than non-transformed MCF10A and 184B5 cell lines (Fig.R1). Moreover, GRK2 protein levels were also up-regulated in MDA-MB-468 cells that, although usually classified in the "basal A" subgroup, share expression of some luminal epithelial markers and present a tumoural behaviour more similar to that observed in the luminal phenotype (Antoon et al., 2012; MacDougall & Matrisian, 2000; Neve et al., 2006). Conversely, GRK2 protein levels displayed by basal B breast cancer-derived MDA-MB231, MDA-MB157 and Hs578T cells were no different from those detected in normal mammary cells (Fig. R1). These results suggested that GRK2 would be increased in "luminal-like" breast cancers.

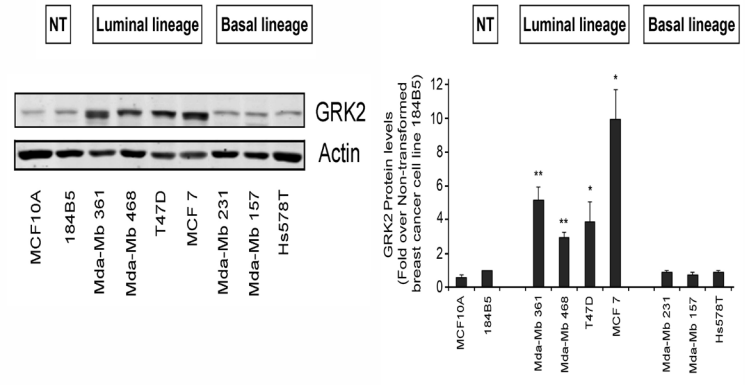


Figure R.1. GRK2 levels are increased in "luminal-like" human breast cancer cell lines. GRK2 expression levels were determined in non-transformed (NT), "luminal-like" and "basal-like" transformed breast cancer cells by western blot with an anti-GRK2 specific antibody. A representative blot is shown and data (mean ± SEM; n = 3) of protein levels relative to the amount of GRK2 in the 184B5 cell line are graphically represented. (*p< 0.05; **p< 0.01)

We searched for potential correlations among such altered GRK2 levels and other features of these transformed breast cells. Mdm2 levels are known to be up-regulated in all oestrogen receptor-positive tumours, which represent two-thirds of all breast cancers, and in 40-80% of metastatic breast tumours in advanced-stage (Araki et al., 2010; Lacroix et al., 2006). Therefore, it is not surprising that all cell types analyzed in our panel display increased levels of MDM2 (Fig. R2A). Up-regulation of GRK2 concurs with aberrant activation of AKT as compared to non-tumoural cells (Fig. R.2B), consistent with either mutational activation of PiK3CA or inactivation of PTEN in these cell types (Table1), thereby suggesting a connexion between enhanced levels of GRK2 and the PI3K/AKT axis. Interestingly, these mutations are not displayed by any of the basal cells analysed in our panel except for MDA-MB468 cells, that shows increased GRK2 expression. Additionally,

it seems that over-expression of GRK2 is irrespective of the p53 levels (Fig. R2C) or the p53 status, since luminal cells of our panel display all the p53 genetic situations present in breast cancer (wild-type p53, p53 null and mutated p53 in MCF7, MDA-MB-361, and MDA-MB-468 and T47D, respectively, see Table 1.

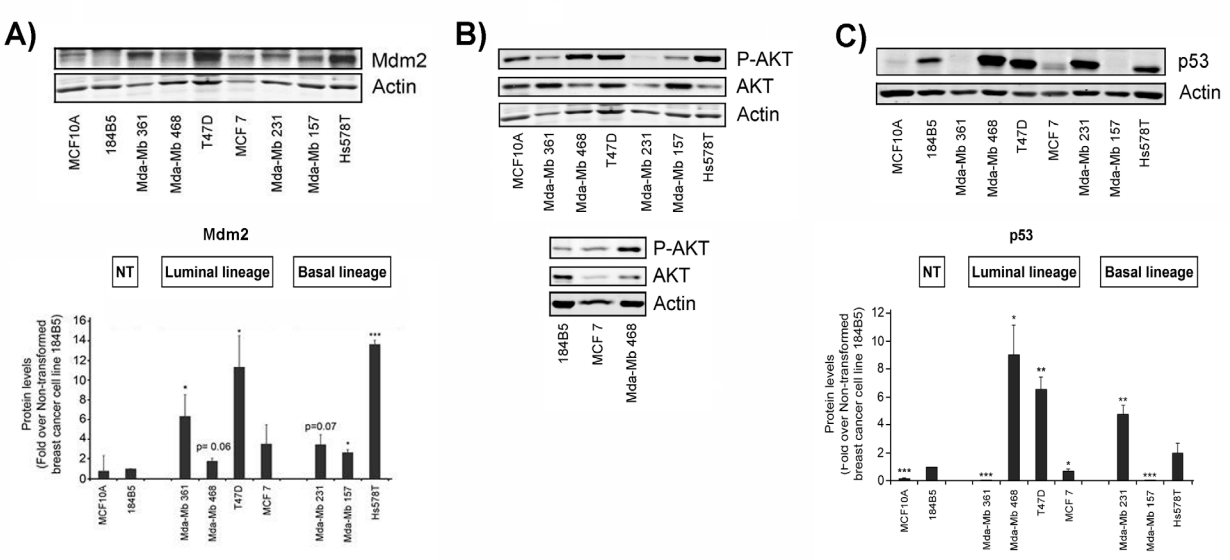


Figure R.2. GRK2 enhanced levels are independent of the Mdm2 or p53 status but correlate with aberrant AKT activation in luminal transformed breast cancer cells. Analysis of Mdm2 protein expression (A), the Ser473-phosphorylated AKT and pan-AKT levels (B), and p53 levels (C) in cell lysates of the indicated breast cancer cell lines by means of immunoblotting with specific antibodies. Representative blots from three independent experiments are shown and Mdm2 values (mean ± SEM) are graphically represented, relative to the amount of Mdm2 in the 184B5 cell line. (*p< 0.05; **p< 0.01; ***p< 0.001).

	MCF10A	184B5	Mda-Mb 361	Mda-Mb 468	T47D	MCF 7	Mda-Mb 231	Mda-Mb 157	Hs578T
ER	-	+	+	-	+	+	-	-	-
PR	-	-	+	-	+	+	-	-	-
HER2 High levels	-	-	+	-	-	-	-	-	-
EGFR High levels	-	-	-	+	-	-	-	-	-
p53mut	-	-	Null	+	+	-	+	Null	+
PiK3CA mut	-	-	+	-	+	+	-	-	-
PTEN mut	-	-	-	+	-	-	-	-	-

Table1. Molecular characterization of oncogenic pathways in the breast cancer cell lines used in this study. Signalling mutated genes and molecular features of normal and breast cancer cell lines used in Fig.R.1. [(+), presence; (-), absence; (null), homozygous deletion].

2.

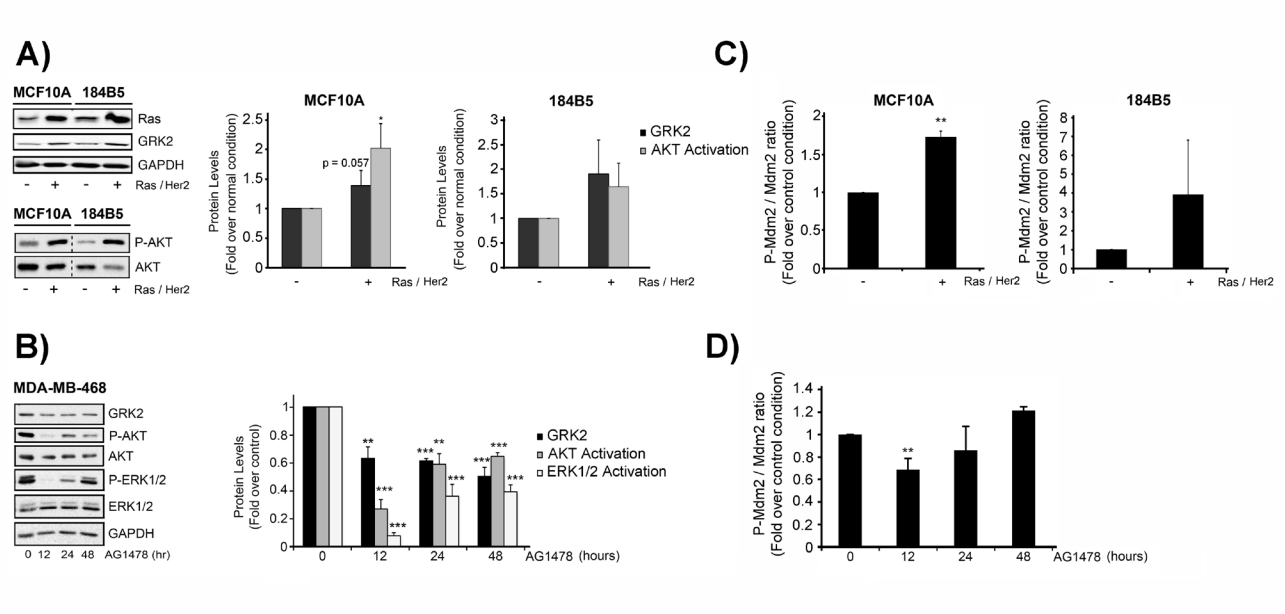
Oncogenic signalling governing breast cancer tumorigenesis up-regulates GRK2 in cellular and animal models.

We sought for additional molecular changes responsible for the up-regulation of GRK2 expression levels. We noted that GRK2 up-regulated cells displayed amplification of receptor growth factors EGFR and HER2 and/or were ER and PR positive (Table1). Notably, all these receptors are able to trigger downstream activation of the PI3K/Akt cascade (She et al., 2008; Sun et al., 2001) (Fig. I.3). Therefore, we decided to explore whether these pathways modulated GRK2 expression.

2.1 Tyrosine kinase receptors are key modulators of GRK2 expression levels.

As shown in Figure R.3A, co-transfection of HER2 and the oncogenic Ras-V12 mutant, known to cooperatively induce the cellular transformation of mammary cells, triggered increased GRK2 protein in non-malignant MCF10A and 184B5 cells in parallel to AKT activation, whereas pharmacological blocking of the EGFR signalling (using the EGFR inhibitor AG1478) markedly reduced the high levels of GRK2 expressed by the

EGFR-overexpressing MDA-MB468 cells in parallel to decreased AKT activation (Fig. R.3B). Moreover, and consistent with our previous data showing that AKT activation blocks Mdm2-dependent GRK2 degradation through the phosphorylation of Mdm2 at S166 / 186 (Salcedo et al., 2006), we found enhanced phosphorylation of Mdm2 at these residues in non-transformed breast cells transfected with the oncogenes Ras/neu (Fig. R.3C), whereas the opposite occurs upon EGFR inhibition in MDA-MB-468 cells (Fig. R.3D).



FigureR.3. GFR-dependent tumoural transformation alters the cellular content of GRK2. A) Non-transformed breast cell lines (MCF10A and 184B5) co-transfected with the oncogene v-Ras and Her2 or with an empty vector (-) were immunoblotted with specific antibodies to measure GRK2 protein levels and the degree of AKT activation. B) The activation extent of the epidermal growth factor receptor (EGFR) correlates with GRK2 expression levels in MDA-MB-468 cells. Cells were treated with the selective inhibitor of EGFR AG1478 (500nM) for the indicated times. GRK2 protein levels and ERK1-2 or AKT activation were assessed in total cell extracts by western blot. C-D) Mdm2 phosphorylation at S166/186 was measured as in section A (C) or B (D). A-D) Data (mean ± SEM of 3 independent experiment) were expressed as the fold induction over control condition. (*p< 0.05, **p< 0.01 and ***p< 0.001).

In addition, we found GRK2 expression specifically increased in those mammary glands of transgenic MMTV-HER2 mice that spontaneously develop tumours, lasting unaltered in the remaining healthy glands of the same mice (Fig. R.4A). Interestingly, tumoural mammary glands also display higher activation of AKT compared to normal ones, pointing at an in vivo correlation between GRK2 expression and AKT activity. Such correlation was confirmed (Fig. R.4B) in mammary glands of transgenic mice expressing myr-AKT, a construct that is constitutively bound to plasma membrane and resembles the pathological localization of cancer-associated AKT mutations (Blanco-Aparicio et al., 2007). Notably, Mdm2 protein levels were also up-regulated in the mammary gland of these mice (Fig. R.4B).

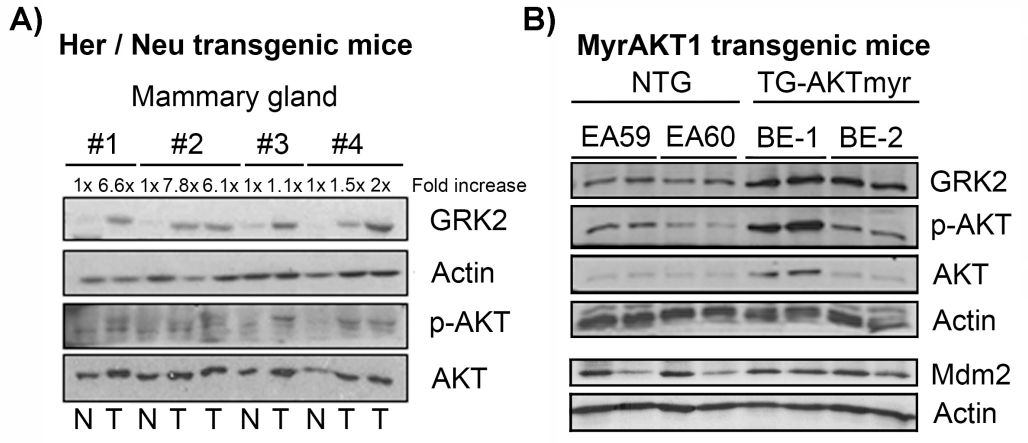


Figure R.4. Concurrent increases of GRK2 protein levels and AKT activation in the mammary glands of transgenic mice lines with enhanced spontaneous breast tumourigenesis or predisposition to develop tumours. Total protein extracts were obtained from tumour-bearing and normal counterparts mammary glands of Her2-transgenic females (mice, n=4) (A) or from glands of MyrAKT1-transgenic and non-transgenic littermate mice as a control (n=2 per group) (B). Tissular proteins were analyzed by western blot to determine GRK2 and Mdm2 expression levels and AKT activation using specific antibodies. Fold-stimulation of GRK2 levels normalized by actin expression over non-tumoural mammary glands is shown.

2.2 GRK2 as a target of the oestrogen pathway

Oestrogen withdrawal for 48-72 hours promoted a notable decrease of GRK2 protein levels in both MCF7 and T47D oestrogen receptor-positive cells (Fig. R.5A). Conversely, chronic exposure of these cells to oestrogen caused an increase of circa 2-fold after 5 days of treatment (Fig. R.5B). Interestingly, the time course of GRK2 accumulation was similar to that of HDAC6 or Mdm2 (Fig. R.5C and D), known transcriptional responders of oestrogen action (Okoro et al., 2013; Saji et al., 2005a).

It is well established that ERα-positive breast tumours respond to tamoxifen treatment. Hence, we sought to determine whether tamoxifen could reverse the oestrogen-promoted accumulation of GRK2 to confirm the oestrogen dependence. Surprisingly, we found that tamoxifen treatment of T47D or MCF7 cells for several days did not modify the levels of GRK2 (Fig. R.6A). However, tamoxifen did not decrease HDAC6 expression levels either (Fig. R6B), what led us hypothesize that our transformed breast cancer cells could have acquired tamoxifen resistance. In fact, GRK2 and HDAC6 protein levels were reduced upon 4 days of low (1uM) or high (5uM) doses of tamoxifen treatment in the oestrogen receptor-positive non-transformed 184B5 cells (Fig. R6B). Collectively, our data indicate that oestrogens directly control GRK2 expression in breast cells.

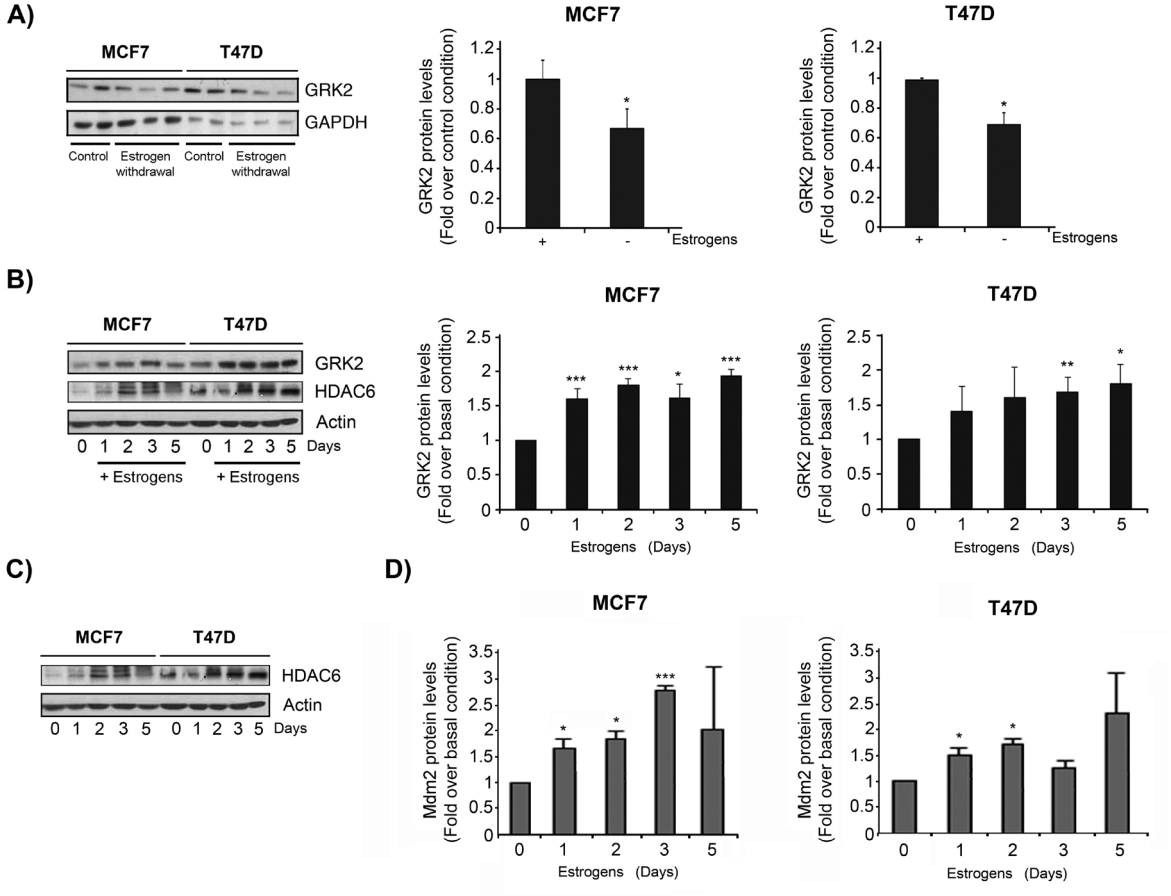


Figure R.5. Expression of GRK2 in luminal breast tumour cells is under control of oestrogens. Oestrogen-receptor positive MCF-7 and T47D cells were grown in serum-complete culture medium (control condition) or phenol-free medium supplemented with charcoal-treated serum for 48-72 hours (oestrogen-depleted condition) (A), or upon oestrogen withdrawal cells were treated with 20nM 17-β-estradiol for the indicated times (B-D). Cell lysates were analyzed for expression of GRK2 (A and B), HDAC6 (C) and Mdm2 (D) by western blot using specific antibodies. A-D) Data (mean ± SEM of 4 independent experiment) were expressed as the fold induction over control condition. (*p< 0.05; **p< 0.01; ***p< 0.001).

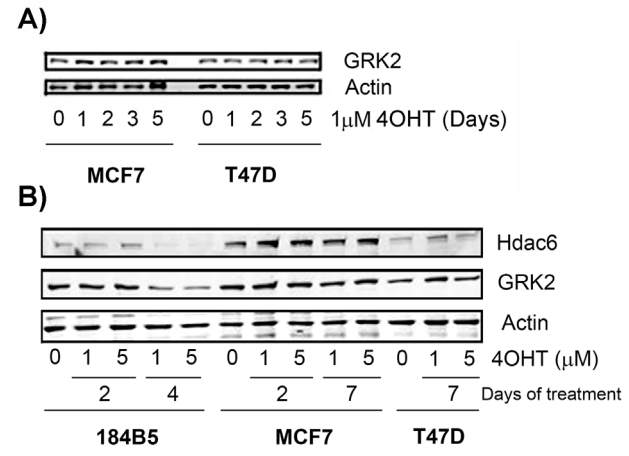


Figure R.6. GRK2 protein levels decay with the anti-oestrogenic tamoxifen in non-transformed sensitive mammary cells, but not in transformed tamoxifen-resistant cell lines. 184B5, MCF7 and T47D cells were first grown in oestrogen-depleted medium for 48-72 h and then treated with the oestrogenic antagonist tamoxifen (1μM or 5 μM) for the indicated times. GRK2 protein levels were assessed by Western Blot using actin as loading control and HDAC6 as a positive read-out of the tamoxifen-dependent modulation of protein expression. Representative western blots are shown.

3.

GRK2 is key to control the p53/Mdm2 axis through the regulation of Mdm2 stability, localization and functionality.

Overall, our results indicate that GRK2 can be upregulated in mammary tumoural models as a result of the activation of known key drivers of breast cancer transformation, including, growth factor receptors, oestrogens or genetic alterations in the PI3K/AKT axis. We suggest that the converging stimulation of Akt by all these pathways would disrupt the normal Mdm2-dependent GRK2 degradation, leading to increased kinase stability and levels. This outcome would occur even in the presence of higher Mdm2 levels in such situations, since growth factor signalling via tyrosine kinase receptors and Ras leads to the activation of AP-1 and Ets family members, which have been shown to transcriptionally up-regulate Mdm2 (Manfredi, 2010), and Mdm2 is a direct target for the oestrogen pathway, contributing to tumour growth and survival (Brekman et al., 2011; Okoro et al., 2013). In fact, concurrent higher Mdm2 and GRK2 levels are observed in the luminal cells of our panel (Fig. R2), in response to oestrogens (Fig. R5) or in the mammary glands of myr-AKT transgenic mice (Fig. R.4B). Since we have reported

that Mdm2 and GRK2 can directly interact (Nogues et al., 2011), we decided to explore whether a different type of functional interaction could be taking place between these two proteins in tumoural contexts.

The regulatory loop between Mdm2 and p53 has been determined crucial to maintain the required levels of p53 and ensure proper cell cycle progression and subsequently the maintenance of cell homeostasis. The Mdm2 gene is one of the targets activated by the transcription factor p53, whereas Mdm2 protein inhibits p53 activity by controlling p53 stability, localization and translation, thus giving rise to a negative feedback loop (Vousden & Prives, 2009). Moreover, Mdm2 also defines regulatory loops with other substrates such as MdmX, in which MdmX interacts and stabilizes Mdm2, increasing its action towards p53, whereas Mdm2 mediates MdmX ubiquitination and turnover (Wade et al., 2010). We hypothesized that the co-existence of increased levels of Mdm2 and GRK2 in luminal-like breast cancer cells could reflect a functional cooperation between these factors in order to confer resistance to growth arrest and to apoptosis, as suggested by the fact that GRK2 attenuates the DNA damage-induced increase of p53 in the context of cell cycle (Penela et al., 2010b). Therefore, we explored whether GRK2 and Mdm2 could define a new regulatory loop, with a possible impact on p53 responsiveness and perhaps on other signaling nodes.

3.1 GRK2 increases Mdm2 stability in a kinase-independent manner

Post-transcriptional modifications in Mdm2 are key events in the regulation of Mdm2 functionality (Coutts et al., 2009) and p53 responsiveness. Some of these modifications involve the direct binding of different partners with Mdm2, modifying E3 ligase auto-ubiquitination and stability (Lee & Lozano, 2006). Based on our previous results of a direct Mdm2 and GRK2 interaction, (Nogués et al., 2011), we analyzed the effect of increasing GRK2 protein or activity levels on the stability of Mdm2. To investigate the role of GRK2 in Mdm2 turnover, pulse-chase assays were performed in HEK-293 cells transiently transfected with Mdm2 in the presence of wild type GRK2 or the catalytically inactive GRK2-K220R mutant. In control conditions, the half-life of Mdm2 was estimated to be of circa 15 minutes in line with other reports (Finlay, 1993). However, in the presence of wild-type GRK2, the protein decay of Mdm2 was notably retarded (~4-fold increase in half-life). Interestingly, a similar trend was noted when the catalytically inactive mutant of GRK2 (GRK2-K220R) was present (Fig. R.7), pointing to a scaffold effect in Mdm2 stabilization, either by favoring a ligase conformation that reduces auto-ubiquitination or altering the recruitment of factors required for Mdm2 degradation.

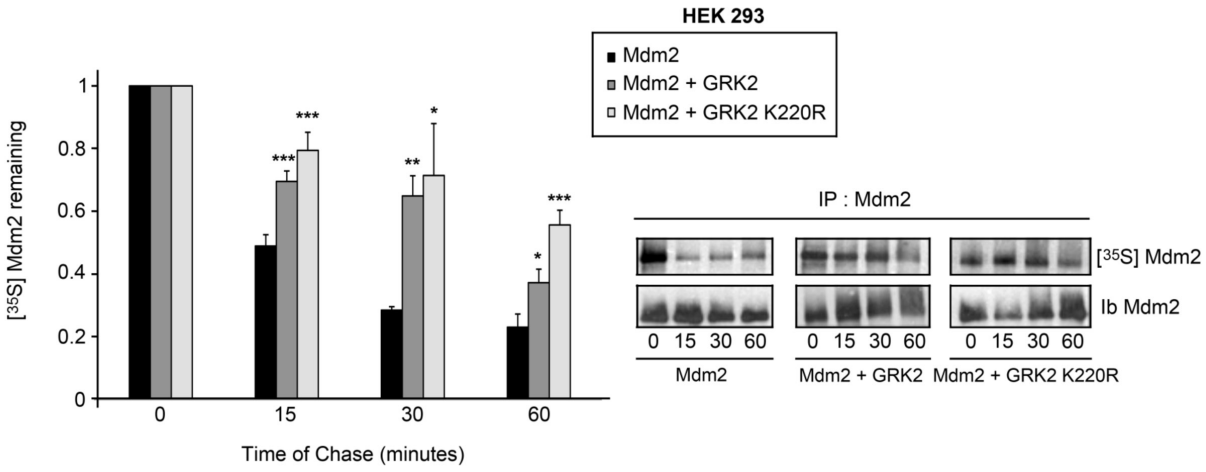


Figure R.7. GRK2 increases Mdm2 stability in a kinase-independent manner. HEK-293 cells were co-transfected with wild-type Mdm2 in the presence or absence of GRK2wt activity or the catalytically inactive mutant GRK2-K220R. Degradation of the Mdm2 protein was assessed by pulse-chase experiments as described in Materials and Methods. 35S-labeled proteins immunoprecipitated with the anti-Mdm2 antibody were resolved by SDS–PAGE followed by fluorography and densitometry. 35S-labeled Mdm2 band densities were then normalized to total Mdm2 present in the immunoprecipitates. Data are mean ± SEM of at least 4 independent experiments performed in duplicate. A representative blot is shown (*p< 0.05; **p< 0.01; ***p< 0.001).

3.2 GRK2 increases the basal association of Mdm2 with p53, resulting in a reduction of p53 steady-state levels.

We next investigated whether such effects of GRK2 levels and activity on Mdm2 levels had an impact on Mdm2-mediated regulation of p53. We initially utilized the HEK293 cell line, which displays wild-type p53. GRK2 wild-type or the catalytically inactive mutant GRK2-K220R were co-transfected with Mdm2 and with or without extra p53 and total levels of Mdm2 and Mdm2/p53 complexes were measured. Interestingly, both GRK2 constructs tended to increase the basal steady-state levels of Mdm2 (Fig. R.8A), in line with our data above, although the wild-type form of GKR2 was more efficient. Notably however, only GRK2 wild-type was able to significantly increase Mdm2-p53 association in either endogenous or over-expressed p53 conditions (Fig. R.8B). In line with this, only the presence of extra wild-type GRK2 significantly decreased p53 levels or potentiated the effect of over-expressed Mdm2 on this parameter in HEK-293 cells (Fig. R.8C).

Taken together, these data indicate that, even though the inactive mutant of GRK2 could increase Mmd2 expression levels, only the wild type form GRK2 could promote an effect on p53 protein expression.

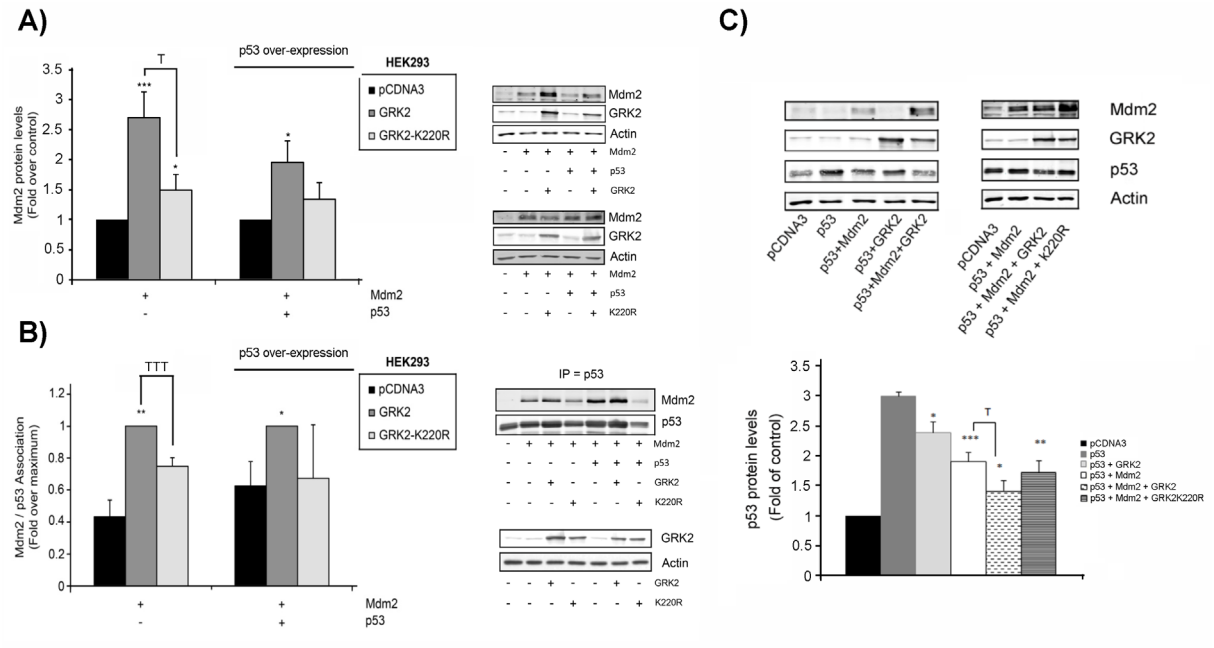


Figure R.8. GRK2 kinase activity potentiates Mdm2 functionability towards p53. HEK-293 cells were transiently transfected with different combinations of p53, Mdm2 and wild type or GRK2-K220R constructs. A) Whole cell lisates were analyzed by immunoblotting with anti-GRK2 and anti-Mdm2 specific antibodies and normalized to levels from control. Data (mean ± SEM of 3-10 independent experiments) and representative blots are shown. B) p53 and Mdm2 interaction was assessed immunoprecipitating p53 with anti-p53 (DO1) antibody coupled to agarose beads and analysed by western blot. To compare the association of Mdm2 to p53, blots were normalized taking the maximum Mdm2/p53 association as a 1. Data (mean +/- SEM of 2-6 independent experiments) and representative blots were shown. C) GRK2 potentiates p53 turnover in a kinase-dependent manner. Whole cell lisates were analyzed by immunoblotting with specific antibodies. The amount of p53 in each condition was normalized to control condition. Data (mean ± SEM of three independent experiments) and representative blotts are shown. (* or ^{T} p< 0.05; **p< 0.01; *** or ^{TTT} p< 0.001).

3.3 GRK2 prevents p53 stabilization upon genotoxic treatment by increasing Mdm2 protein stability.

Under normal circumstances, p53 is maintained at very low levels by continuous ubiquitination and degradation. Genotoxic stress triggers de-phosphorylation of several Ser/Thr residues in the acidic domain of Mdm2 in order to protect p53 from degradation (Zhang & Prives, 2001). Additionally, DNA damage induces ATM-dependent phosphorylation of Mdm2 at S395 (Cheng et al., 2011; Maya et al., 2001). The consequence of these modifications is to prevent poly-ubiquitination of p53 and to promote its rapid stabilization. Therefore, we next sought to establish whether GRK2, through the modulation of Mdm2, could alter p53 accumulation upon DNA damage. Stable expression of extra levels of GRK2 in non-transformed mammary 184B5 cells prevented p53 protein accumulation upon doxorubicin treatment (Fig. R. 9A). Given that ubiquitination and protein degradation (mainly by the ubiquitin ligase Mdm2) is a pivotal process in down-regulating p53 functions (Shi & Gu, 2012), we next analyzed whether GRK2 altered p53 turnover under cellular stress. The stability of p53 was determined in cells expressing extra

GRK2 (185B5-GRK2) or control cells (184B5) upon doxorubicin-promoted DNA damage using a cycloheximide chase assay. After doxorubicin challenge, cycloheximide was added to inhibit de novo p53 synthesis and the steady-state levels of p53 were detected. As shown in Figure R.9B, the levels of p53 protein decline slowly in control cells with a half-life of ~4h, while in the presence of extra GRK2 the half-life was decreased ~2-fold. Conversely, Mdm2 decay was alleviated in GRK2 over-expressing 184B5 cells, consistent with the GRK2-mediated stability effect on Mdm2 in 293 and MCF7 (Fig R.9C and R.7-8). To rule out the possibility that prolonged doxorubicin treatment (up to six hours) could cause some changes in protein expression, 184B5 cells were treated for several hours with doxorubicin without adding cycloheximide. As shown in figure R.9D and B, p53 levels were induced at 1 hour of doxorubicin treatment and maintained high all along the experiment in a similar way to GRK2 protein levels. These also verify recent results from our group showing that doxorubicin treatment are able to trigger GRK2 stabilization, which correlated with the extent of induction of p53 and apoptosis in the G2 checkpoint response (Penela et al., 2010b). Moreover, Mdm2 protein levels were also up-regulated, likely as a combined result of the transcriptional action of p53 and the new role herein described for GRK2 (Fig R.9D). Overall, these data are consistent with a role for GRK2 in favoring the degradation of p53 under stress conditions.

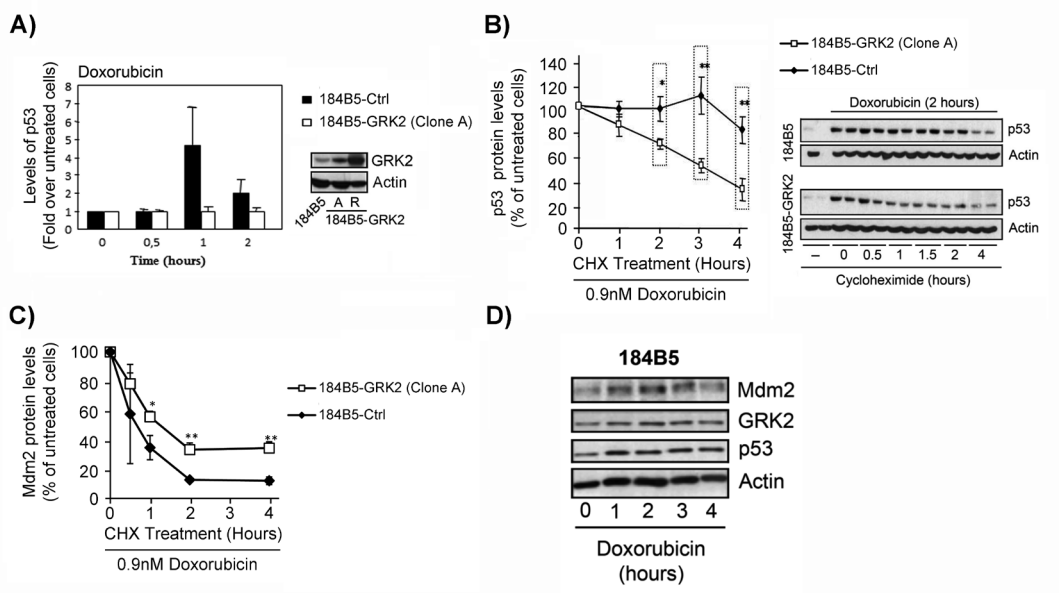


Figure R.9. GRK2 prevents p53 stabilization upon genotoxic treatment by increasing Mdm2 protein stability. A) Parental and stable 184B5 cells that over-express GRK2 were exposed to doxorubicin (0.9nM) for the indicated times. p53 protein induction was measured by western blot. Data (mean ±SEM) from 3 independent experiments are shown. B-C) Parental and stable 184B5 cells that over-express GRK2 were treated with doxorubicin (0.9nM) for two hours to induce upregulation of p53. Then, cycloheximide (20μg/ml) was added to cells for the indicated periods. Cells were lysed and total p53 (A) and Mdm2 (B) levels were determined by immunoblotting. Protein levels were normalized and depicted as percentage of expression at 0 hours of cycloheximide treatment. Data (mean ± SEM.) from 4 independent experiments are shown (*p<0.05, **p<0.01). D) 184B5 cells were exposed to a doxorubicin treatment (0.9nM) for several hours. Cell lysates were analyzed then by western blott with specific antibodies. A representative western is shown.

3.4 GRK2 promotes the nuclear shuttling of Mdm2 in a kinase dependent manner.

It was assumed that the cytoplasm is the exclusive site of p53 degradation, however it is now evident that the nucleus constitutes also a significant proteasomal compartment for MDM2-mediated degradation with relevant physiological implications (Shirangi et al., 2002). Thus, nuclear shuttling of Mdm2 is needed for p53 to be down-regulated quickly when DNA damage is repaired in normal cells and cellular homeostasis must be resumed. Survival signals contribute to switch the p53 pathway off in this context by triggering the nuclear import of MDM2 via AKT activation and ligase phosphorylation. As in tumoural contexts Mdm2 displays altered subcellular localizations, we next explored the potential effect of GRK2 on Mdm2 nuclear-cytosolic shuttling, as another posttranscriptional-modified variable relevant for Mdm2 function and the output of p53 responsiveness. (Jackson et al., 2006; Ogawara et al., 2002).

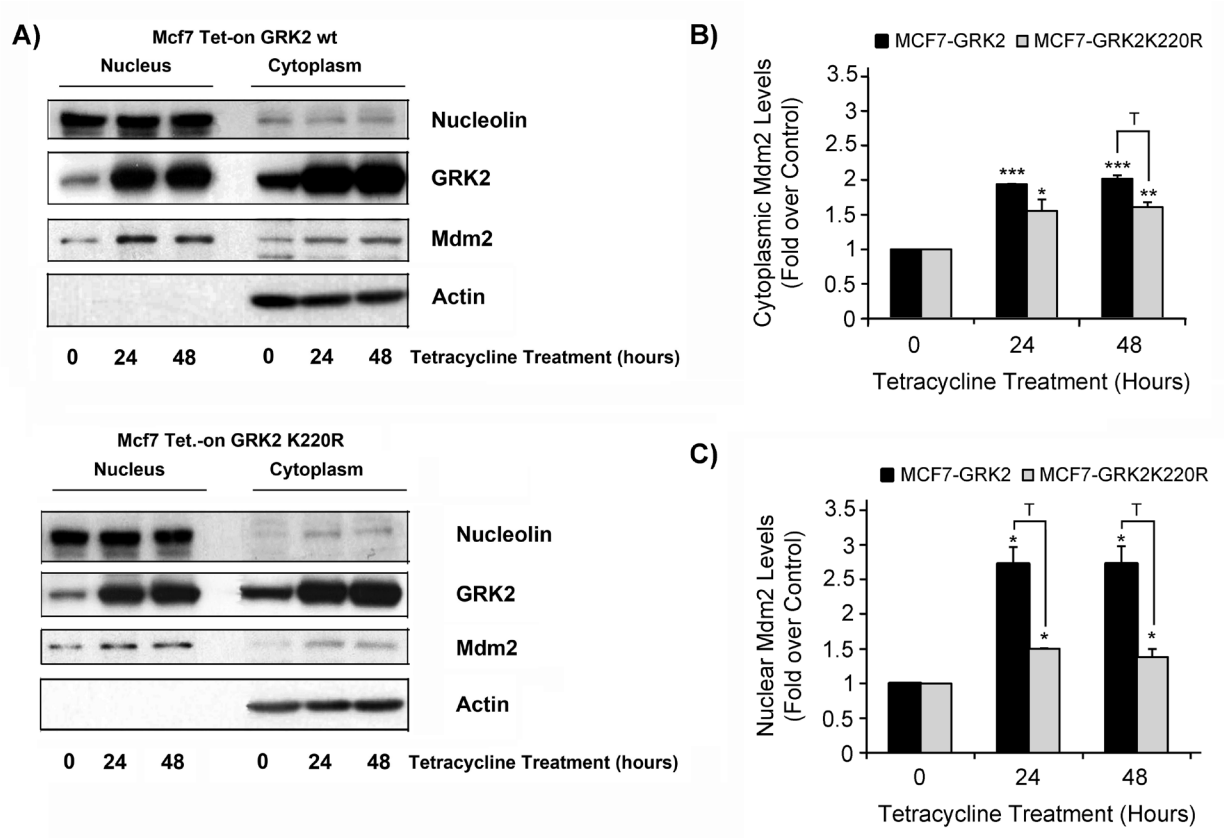


Figure R.10. GRK2 promotes nuclear shuttling of Mdm2 in a kinase-dependent manner. A) Lysates from MCF7 cells were subcellularly fractionated upon tetracyclin-inducuble expression of wild-type GRK2 or mutant GRK2-K220R for the indicated times. The protein levels of GRK2, Mdm2 and p53 were analyzed in cytoplasmic and nuclear fractions with specifics antibodies. Nucleolin and Actin content was used to monitor the accuracy of cellular fractionation and as loading controls. B-C) Cytoplasmic (B) or nuclear (C) Mdm2 expression data (mean ±SEM from 3 independent experiments) were plotted. (* or T $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$)

In order to establish this, we generated MCF7 breast cancer cells engineered using the TET-on system to timely induce the expression of wild-type GRK2 or its catalytically inactive mutant GRK2-K220R in the presence of tetracycline. After 24 and 48 hours of tetracycline treatment, a sub-cellular fractionation was performed and endogenous Mdm2 protein levels analyzed in both nuclear and cytoplasmic compartments. Consistently with our previous results in 293 cells, GRK2 or K220R increased Mdm2 expression levels in the cytoplasmic fraction of MCF7 cells in a kinase activity-independent manner (Fig. R.10A and B). However, we cannot discard a kinase dependent component since Mdm2 levels are significant higher at 48 hours in the presence of wild-type GRK2. Interestingly however, GRK2 promotes a marked nuclear re-localization of the ligase in a way enhanced by its kinase activity (3-fold increase for wt vs 1.5-fold in the presence of K220R) (Fig. R.10A and C).

4.

GRK2 confers resistance to diverse genotoxic insults.

Both intrinsic and acquired resistance to cell death is a common and important feature of cancer progression that relies on the attenuation of p53 functions. Growth factors, tyrosine kinase receptors, and certain oncogenes have been shown to be effective inhibitors of apoptosis, and in many cases, their anti-apoptotic effects are mediated by the PI3K-induced activation of Akt that promotes Mdm2 nuclear shuttling and Mdm2-mediated ubiquitination and degradation of p53 protein (Datta et al., 1999; Ogawara et al., 2002). Since we shown herein that GRK2 expression is up-regulated in different luminal like breast cancer cells and that the levels of this kinase can alter Mdm2 levels and Mdm2-mediated p53 turnover upon basal and stress-induced conditions, we explored the potential effect of GRK2 levels in the activation of p53 and apoptotic response upon cellular damage. Although the cellular dosage of p53 protein is the single most important determinant of its function, posttranslational modifications by distinct stress-activated kinases are also relevant for transcriptional activation of p53 target genes, namely ATM-mediated phosphorylation at the p53 transactivation domain (Ser15). This key residue lays on a docking motif for p300 that is required to promote DNA-dependent

acetylation of p53 at promoters and to trigger specific apoptotic functions of p53 (Chao et al., 2003, 2006; Sluss et al., 2004). Stable over-expression of moderate levels of GRK2 protein (184B5-GRK2) strongly attenuates levels of phospho Serine 15 (Fig. R.11A). Moreover, extra levels of GRK2 in non-transformed mammary 184B5 cells also conferred resistance to p53 activation upon cisplatin and doxorubicin treatment, another genotoxic compounds commonly used in chemotherapeutic treatments (Fig. R.11 B and C).

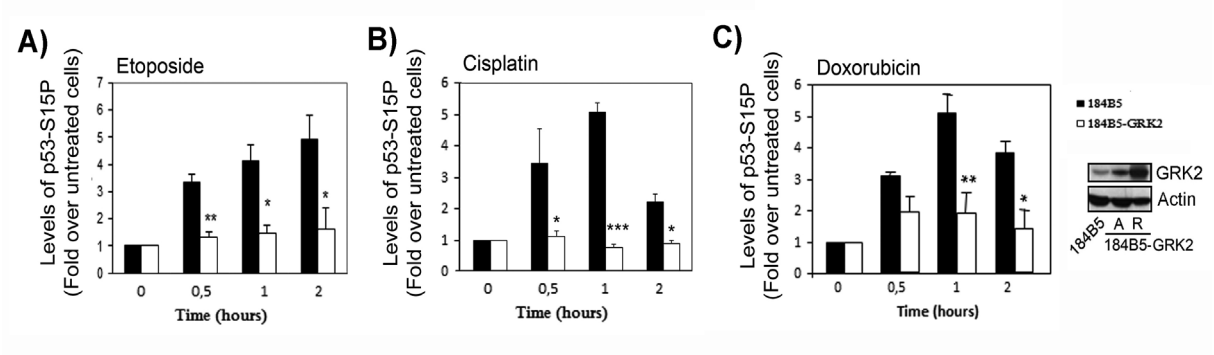


Figure R.11. Over-expression of GRK2 downmodulates the activation of p53 in response to genotoxic damage in non-transformed mammary cells. Parental and stable 184B5 cells that over-express GRK2 were exposed to etoposide (2μM) (A) cisplatin (0.5μM) (B) or doxorubicin (0.9nM) (C) for the indicated times. Phospho-Ser15 p53 levels were determined in cellular lysates by western blot and normalized by total p53 levels. Data (mean ± SEM) of p53 activation from three independent experiments are shown. (*p<0.05, **p<0.01, ***p<0.001 are p values compared to control cells).

Consistent with the GRK2-dependent down-modulation of Ser15 phosphorylation, GRK2 levels correlate inversely with robustness of apoptotic responses. Transformed MCF7 cells were shown to be resistant to apoptosis triggered by the commonly used cytotoxic compounds paclitaxel (a microtubule damage agent) or etoposide (a genotoxic drug), as indicated by the modest decrease in pro-caspase 3 levels, which decay reflects caspase-3 activation (Fig. R.12A). Such response was however apparent upon adeno-shRNA-mediated GRK2 downmodulation (circa 50-70% of procaspase-3 decrease), what suggest that silencing GRK2 expression can sensitize these cells to cellular stresses. A similar trend in pro-caspase7 protein decay is noted in MCF7 cells transfected with a shRNA-GRK2 construct compared to kinase overexpression (Fig. R.12B). Moreover, depletion of GRK2 results in a notable increase of PARP cleavage induced by both etoposide or paclitaxel treatment compared to cells with extra GRK2 levels (Fig. R.12C), which otherwise accumulate less cleaved PARP than parental cells (Fig. R.12D).

Overall, these results suggest that GRK2 could limit the p53 responsiveness, increasing cell resistance to programmed cell death and contributing to cell survival, both relevant hallmarks for tumor progression and important chemotherapeutic-resistant factors.

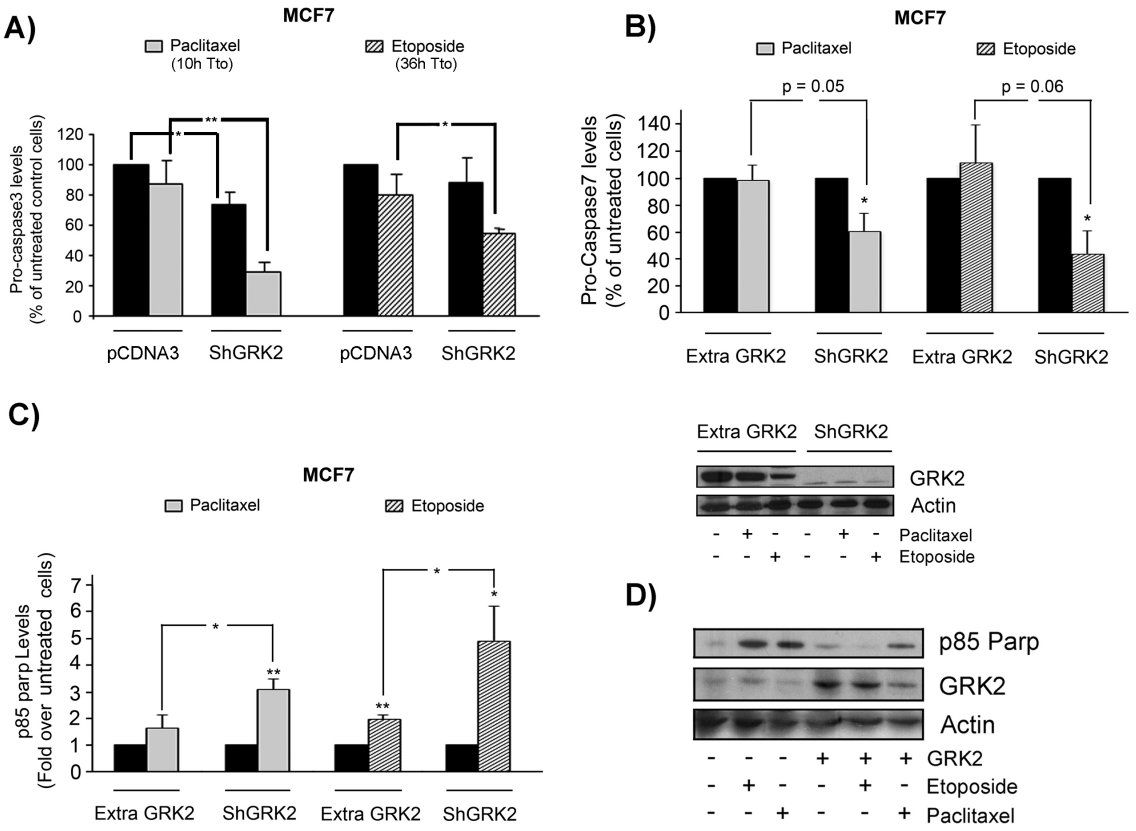


Figure R.12. GRK2 confers cell-death resistance to diverse cytotoxic agents in luminal tumoural breast cells. (A-D) GRK2 levels inversely correlate with pro-apoptotic mediators. MCF7 cells either transiently transfected with the indicated constructs (A,D) or co-transfected with a cDNA encoding for the CD8 antigen (B-C) for subsequent CD8-based selection were treated with paclitaxel or etoposide as detailed in Methods section. Levels of GRK2, pro-caspase-3 (A), pro-caspase-7 (B) or the PARP fragment (C-D) were determined by western blot. Data are mean ± SEM (n= 2-4, *p<0.05, **p<0.01)

5.

GRK2 compromises reactivation of p53 responsiveness in response to Nutlin3a.

Strategies to induce p53 activation in tumors that retain wild-type p53 are promising for cancer therapy. In this context, the ubiquitin system has emerged as the central node for the development of cancer therapeutics focused on p53 induction (Yang et al., 2009). Since E3 ligases play a major role in determining the specificity of ubiquitination, they are regarded as key targets for therapeutic intervention.

Different strategies have been employed to specifically inhibit Mdm2-mediated p53 ubiquitination. Based on the structure of the p53/Mdm2 complex, a number of small molecules, including Nutlins, RITA, MI-63 and SyI-155, have been developed to block the binding of p53 to Mdm2 (Yang et al., 2009). Particularly, Nutlin-3a is a small molecule that binds Mdm2 at the hydrophobic p53 binding pocket within its N-terminus, resulting not only in non-genotoxic stabilization of p53, but also in the stimulation of p53 transcriptional activity (see scheme in Fig R.13A). Hence, Nutlin 3a treatment promotes cell cycle arrest and apoptosis in wild type p53-harboring tumoural cells in culture and in mouse xenografts (Tovar et al., 2006; Vaseva et al., 2009). Moreover, it has been shown that those cell lines that overexpress MDM2 are the most sensitive to Nutlin-3a treatment (Tovar et al., 2006).

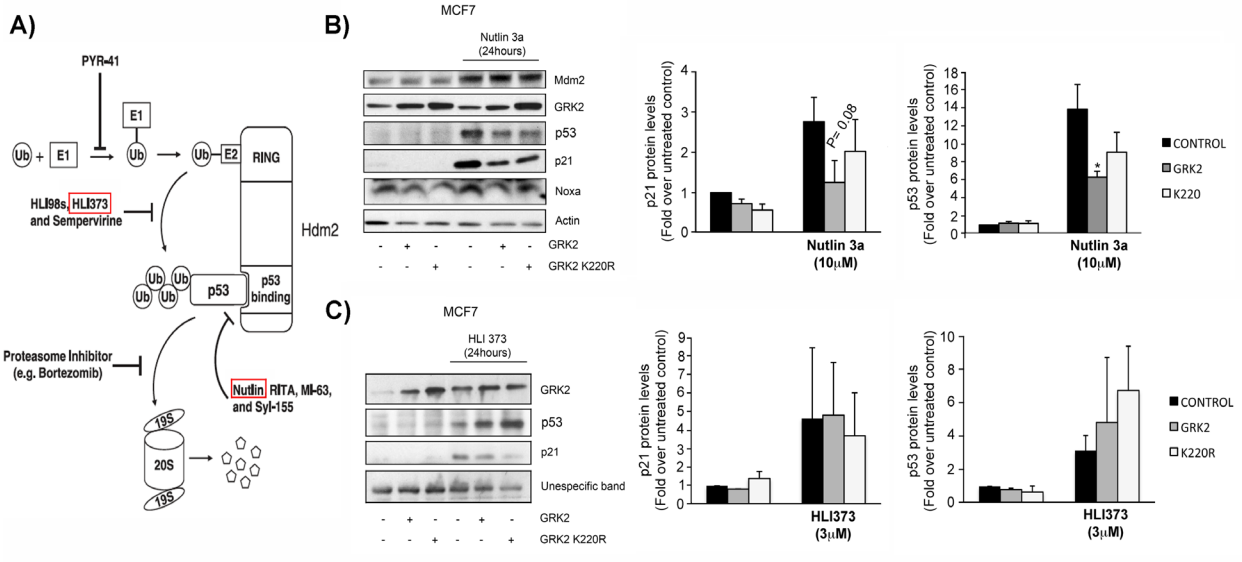


Figure R.13. GRK2 attenuates the Nutlin 3a-induced up-regulation of p53 response in a process that requires Mdm2 ligase activity. A) Cartoon depicting the mechanism of action of Mdm2 inhibitors (specially highlighted Nutlin-3a and HLI373). B-C) MCF7 were infected with adenoviral GRK2 constructs (wild type and mutant GRK2-K220R) or with a control adenovirus. Cells were treated with vehicle, 10μM nutlin3a (B) or 3μM HLI373 (C) for 24 hours, and lysates were analyzed for expression of p21, p53, Mdm2 and Noxa with specific antibodies. GRK2 levels were also examined to confirm equivalent transduction of the different constructs used. Data (mean ± SEM) of four (B) or two (C) experiments are shown. (*p<0.05)

Since enhanced GRK2 levels protect cells from apoptosis upon genotoxic insults and increase Mdm2-p53 interaction, we analyzed the effects of Nutlin3a on p53 activation in cells expressing different GRK2 cellular dosages. As shown in Fig R.13B, Nutlin3a treatment of control MCF7 cells caused up-regulation of the p53 target genes p21 and the pro-apoptotic factor Noxa, along with increased expression of p53 and Mdm2. Interestingly, such reactivation of p53 responsiveness was compromised by over- expression of wild type GRK2 (and not significantly by the K220R mutant). Of note, Mdm2 was upregulated in cells expressing either extra GRK2 or K220R, suggesting that the Mdm2- stabilizing effect of GRK2 occurs independently of p53-mediated transcription of Mdm2. In addition, these results suggest that Mdm2 could be less "drugable" by Nutlin3a in the presence of high GRK2, probably because GRK2 promotes a strong Mdm2/p53 association and reduces Nutlin3a binding. Again, the efficacy of GRK2 action on avoiding p53 induction upon Nutlin3a treatment is higher when the kinase domain of GRK2 remains active, which strongly argues for the existence of both kinase-dependent and independent mechanisms of Mdm2 control by GRK2.

An alternative approach to prevent Mdm2-mediated p53 ubiquitination is by inhibiting the ubiquitin ligase activity of Mdm2. Recently, HLI373 has been identified as a water-soluble inhibitor of Mdm2 ubiquitinase activity (Yang et al., 2009). This compound

also increases p53 levels, induces apoptosis and preferentially kills transformed cells retaining wild-type p53. Thus, we assessed whether GRK2 would also alter the response to this factor. As shown in Figure R.13C, 24 hours-treatment of HLI373 in MCF7 cells promoted an increase of p53 responsiveness, by means of increased levels of p53 and its downstream effector p21. However, this induction was not reverted by the presence of extra levels of either GRK2 wild type or GRK2 K220R, suggesting that GRK2 would require the ubiquitinase activity of Mdm2 to potentiate p53 downmodulation.

Taken together, these data strongly point to GRK2 as a key mediator of apoptosis resistance in wild type-p53 breast cancer cells, by inhibiting the induction of p53 response upon genotoxic and Nutlin3a treatments. The mechanism underlying these events seems to involve the GRK2 dependent modulation of Mdm2, since the E3 activity of the ligase is necessary to promote GRK2-mediated p53 turnover in such situations. Moreover, these results also put forward that the impact of GRK2 in DNA damage-dependent activation of p53 could be routed independently of the ATM-CHK2 module, since Nutlin3a stabilizes p53 without requiring its phosphorylation by ATM or related DNA damage activated kinases (Efeyan et al., 2007).

6.

GRK2 phosphorylates Mdm2 in its acidic domain and nearby its nuclear localization sequence.

The data above indicated that this novel regulatory role of GRK2 in p53 responsiveness might rely on kinase activity-mediated effects on different mechanisms that control Mdm2 stability, localization and/or Mdm2-dependent degradation of p53. Since we had previously reported that Mdm2 and GRK2 proteins interact in a direct manner (Nogués et al., 2011), we explored the possibility that Mdm2 could be a GRK2 substrate. Preliminary experiments in our lab showed that GRK2 phosphorylated Mdm2 in a specific way and with high affinity in "in vitro" kinase assays using recombinant GRK2 and purified GST-Mdm2 fusion protein. To further identify the specific sites of Mdm2 phosphorylation by GRK2, we used GST-fusion proteins spanning different regions of Mdm2 as substrates of the kinase assay (see Methods section and Fig. R.14A). As shown in Figure R.14B, full-length Mdm2 was efficiently phosphorylated, while control GST protein was not. Deletion of the N-terminal p53-binding domain (residues 1-100) did not prevent the phosphorylation of Mdm2 by GRK2, whereas the RING-finger domain of Mdm2 (aa 430-491) was unable to be phosphorylated by GRK2. In contrast, an Mdm2 construct bearing residues 100-200

was phosphorylated to some extent. These results could mean that the phospho-acceptor sites would reside mainly on the central region of the ligase (residues 200-430), which displays important determinants for the interaction with a plethora of Mdm2 partners, but did not rule out that the 100-200aa region would harbor specific phosphorylation sites targeted by GRK2, and that the poor phosphorylation observed was due to an inefficient docking of the kinase to the substrate, supported in the full-length Mm2 by adjacent or accessory regions not present in the Mdm2100-200 construct.

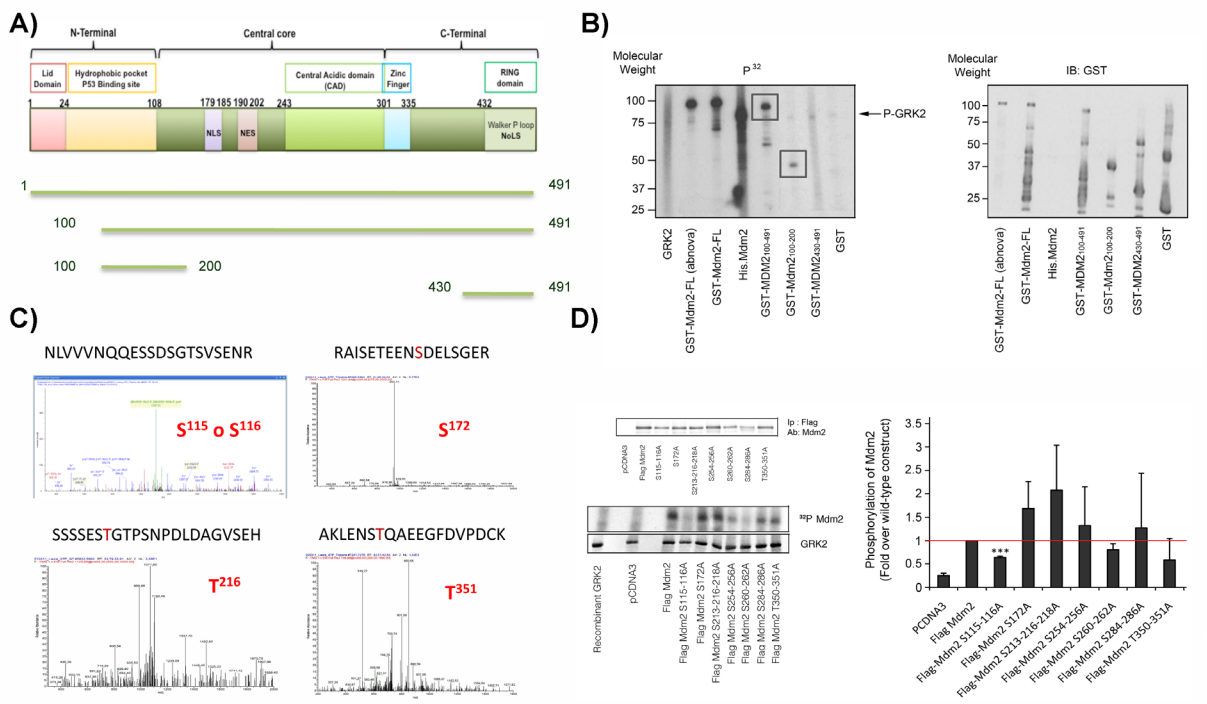


Figure R.14. Identification of GRK2-mediated phosphorylation sites in Mdm2. A) Cartoon depicting the different domains of Mdm2 and the GST-Mdm2 constructs used for the kinase assay. B) GST-Mdm2 constructs,the his-tagged Mdm2 full lenght or GST alone (100nM) were incubated in vitro in the presence of recombinant GRK2 (50nM) and [32P]- ATP, followed by SDS-PAGE and analysis for 32P incorporation by autoradiography. Recombinant GRK2 was also incubated alone as a positive control of autophosphorlylation. The same amounts of the different fusion proteins were analyzed by Western Blot to confirm equal protein loading. C) GST-Mdm2 fusion protein (250ng) was subjected to a protein kinase assay with recombinant GRK2 protein (100nM) in the presence or absence of ATP and then was digested by trypsin and chymotrypsin to obtein the sequence coverage. Candidate peptides were analyzed by LC-MS/MS. Positive peptides and phosphorylation peaks are shown. D) A battery of Flag-Mdm2 constructs displaying single, double, or triple mutations to alanine were generated to target potential serine/threonine residues in Mdm2 phosphorylated by GRK2. Upon cell expression of the indicated constructs, Mdm2 immunoprecipitates were incubated with recombinant GRK2 in the presence of [32P]-ATP as in section B, followed by SDS-PAGE and analysis for 32P incorporation by autoradiography (left pannels) or the presence of comparable amounts of GRK2 by Comassie staining. The presence of comparable amounts of Mdm2 was dedected by immunoblotting with the anti-flag antibody. Data are mean ± SEM of three independent experiments. (***p<0.001).

To further explore this issue we performed proteomic analysis. Mass spectrometry assays, pointed at S115 or S116 and S172 (nearby the nuclear localization and exportation sequence), T216 in the acidic domain and S351 as residues phosphorylated in the presence of GRK2 (Fig. R.14C). In order to confirm these results, a mutagenesis strategy was designed (Fig. MM1 and Fig. R.14D), generating a battery of single, double or triple mutations to alanine in the aminoacids that had been identified in the mass spectrometry assay. Additionally, some alanine point mutations were also generated in several aminoacids within the acidic domain, such as S256 and S260 or S262, which are normally phosphorylated in the cells under non-stressed conditions (Meek & Knippschild, 2003) but appear to be de-phosphorylated under DNA damage conditions, to check a possible opposite effect caused by GRK2-dependent phosphorylation of the E3 ligase. In vitro phosphorylation assays in HEK-293 cells indicated that mutation of residues S115/116 significantly reduced Mdm2 phosphorylation by GRK2, whereas a tendency to diminished phosphorylation was noted upon mutation of S260/262 or T350/351 (Fig. R.14D). These data are consistent with our previous results showing that GRK2 activity promoted nuclear translocation of Mdm2, Mdm2-p53 association and p53 destabilization. Surprisingly, mutations of Mdm2 at S172, S231/T216/T218 or at S254/256 that otherwise prevent phosphorylation by other kinases (CK1, CDK2 or GSK3) (Meek & Knippschild, 2003; Shi & Gu, 2012; Zhang & Prives, 2001) seem to cause enhanced phosphorylation by GRK2.

7.

GRK2 promotes the acquisition of oncogenic properties

Once the effects of GRK2 expression on the Mdm2/p53 axis had been preliminarily characterized, we next explored whether the enhanced levels of GRK2 detected in luminal breast cancer cells would have additional impact in the process of breast tumour proliferation. Notably, the breast cancer cell lines showing enhanced GRK2 levels also displayed increased expression of key markers of cell proliferation (Cyclin D1), mitotic regulation (Pin1) and mitosis entry (phospo-His3) compared to non-transformed cells (Fig. R.15). Thus, we investigated whether GRK2 was able to induce expression of these proteins involved in cell growth in a non-tumorigenic background. Remarkably, transient GRK2 overexpression in either 184B5 or MCF10A non-transformed breast cells promoted a significant increase in the protein levels of the proliferation marker Pin1, to an extent similar to that triggered by Ras/Neu transformation (Fig R.16A), and also led to marked upregulation of the pHis3 proliferation marker (Fig R.16B). Stable overexpression of GRK2 in two different 184B5 clones replicated the same effects (Fig. R.16C).

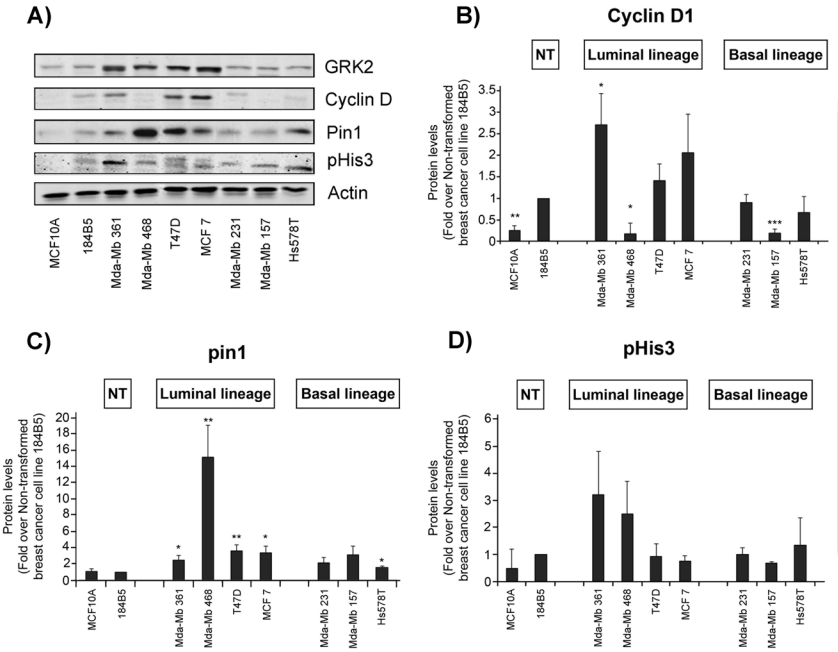


Figure R.15. GRK2 over-expression in luminal breast cancer cells positively correlates with cell-cycle regulators, mitotic markers and proliferation rates. A) Different non-transformed and transformed breast tumour cells were analyzed for the expression levels of proteins involved in growth and survival by Western Blot. A representative gel showing Cyclin D1, Pin1 and phosphoSer10-Histone3 (pHis3) levels is included. B) Protein quantification was expressed as the fold-change over values of non-transformed cell line 184B5 and data (mean ± SEM) are plotted (*p< 0.05, **p<0.01 and ***p< 0.001)

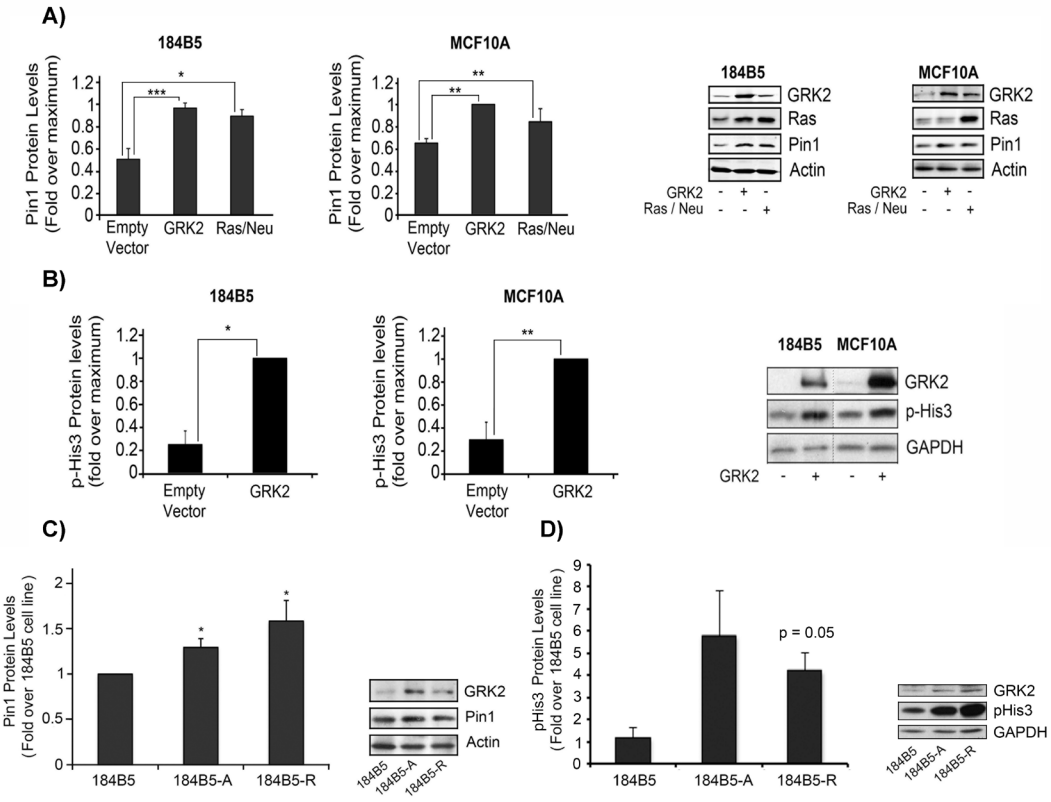


Figure R.16. GRK2 over-expression potentiates the activation of the mitogenic Ras-dependent module in non-transformed mammary cells. (A-B) Up-regulation of the Pin1 (A) and p-Ser10-Histone 3, (pH3,B) mitogenic markers levels in 184B5 and MCF10A cells transfected with the indicated constructs. (C-D) Stable up-regulation of GRK2 at high (184B5-R cells) or moderate (184B5-A cells) levels triggers a sustained increase of Pin1 (C) and pH3 (D) markers. In A-D panels data are mean ± SEM of 3-6 independent immunoblot experiments (*p< 0.05, **p<0.01 and ***p< 0.001, when compared to empty vector-transfected conditions or parental cells).

7.1 GRK2 up-regulation enhances Ras activity in response to EGF

The role of Pin1 in breast tumorigenesis has been widely documented (see Introduction). It has previously shown that Pin1 is a downstream effector of the Ras oncogene (Ryo, 2003; Ryo et al., 2002; Wulf et al., 2001). In addition, stimulation of the Ras-MAPK pathway also leads to phosphorylation of histone H3 (Dunn & Davie, 2005). Thus, although changes in Ras protein levels were not observed in the non-transformed breast cancer cell lines that over-express GRK2 (Fig. R.16), we explored if GRK2 could alter Ras activity to trigger up-regulation of Pin1 and p-His3. Thus, we determined the effect of modified GRK2 levels or activity on the activation of Ras upon EGF treatment by using a Ras GTPase loading assay (see methods). In parental cells, the activation of Ras rapidly peaks at 2 minutes of EGF treatment and then decays (Fig. R.17A). Interestingly, adenovirus-mediated infection of 184B5 cells with a wild-type GRK2 construct increased Ras activation at early times in response to EGF stimulation and prolonged its activated time course, whereas a GRK2-K220R construct did not. The same tendency was also observed using the other model of non-transfomed breast cell line MCF10A (Fig. R.17B). Overall, we can conclude that GRK2 enhances Ras activation by EGF in a kinase-dependent manner.

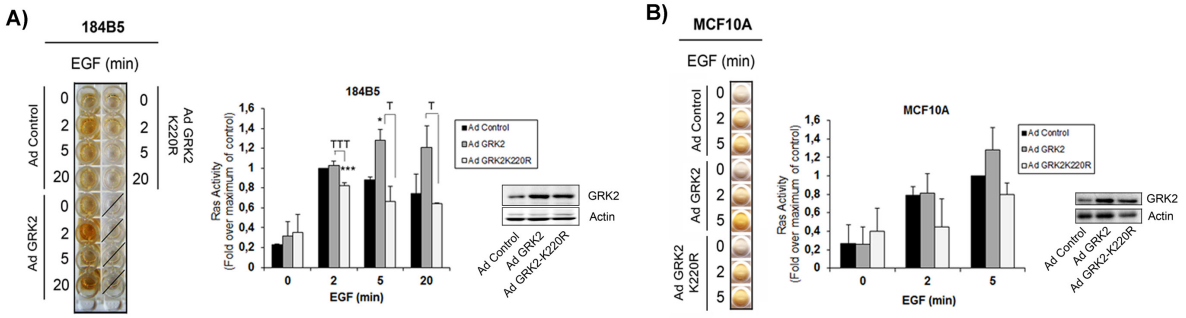


Fig. R17:GRK2 up-regulation enhances Ras activity in response to EGF. Increased GRK2 strengths Ras activation upon EGF challenge in a catalytic kinase-dependent manner. 184B5 cells (A) or MCF10A (B) were infected with adenoviral constructs of wild type GRK2, the kinase-dead mutant (GRK2-K220R) or with a control adenovirus. After 48 hours of infection, cells were serum-starved for 4 hours and stimulated with EGF (100ng/ml) for the indicated times. Ras activity was assessed using the G-LISA Ras Activation Assay Biochem Kit (Cytoskeleton). A representative result is shown and data (mean +/- SEM of 2-3 independent experiments) were plotted as fold-induction over the maximum of the control condition. Statistic analysis are indicated, * or T $p< 0.05$; *** or TTT $p< 0.001$,when compared to control cells (*) or to wild-type GRK2 transduced cells (T).

7.2 GRK2 enhances the activation of both mitogenic and pro-survival signaling in response to potential oncogenic stimuli.

As GRK2 increased the activation of Ras upon EGF treatment, we tested whether GRK2 would increase the activation of signaling pathways controlled by key growth factors receptors in breast cancer. EGF and Heregulin are known agonists of ErbB family of

receptors, which trigger cell growth and survival through the activation of the ERK1/2 and AKT pathways (Citri, 2003; Hynes & Lane, 2005). Interestingly, GRK2 over-expression clearly and significantly facilitated the stimulation of both the mitogenic ERK1/2 and the pro-survival Akt pathways by either Heregulin (Fig. R.18A) or EGF (Fig. R.18B) treatments.

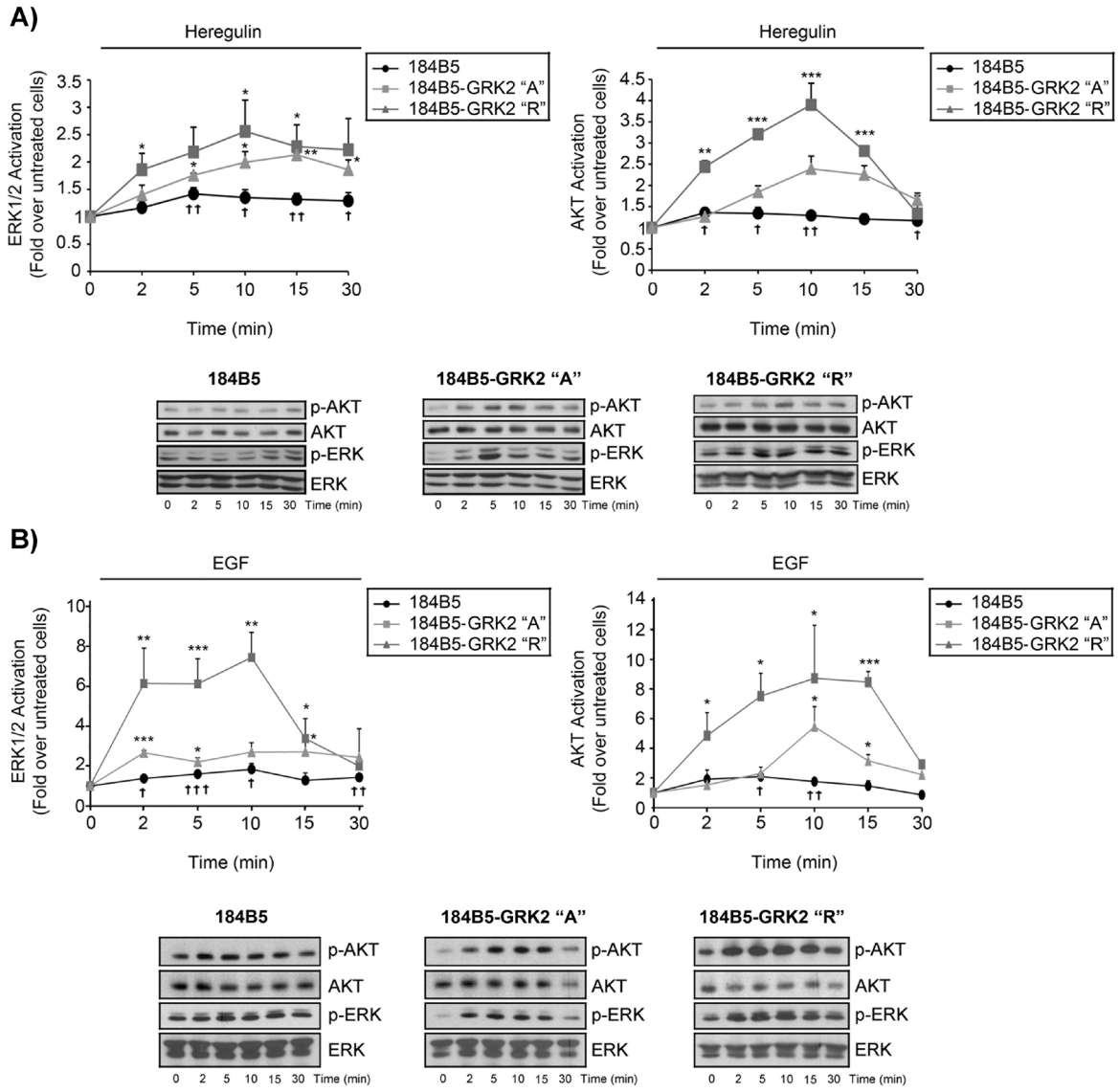


Figure R.18. GRK2 levels positively regulate AKT and ERK1/2 signalling in response to ErbB receptor ligands. Stably GRK2 over-expressing 184B5-A an -R cells and parental 184B5 cells were serum-starved (0.1-1% HS) for 2 hours and stimulated with (A) Heregulin (20ng/ml) or (B) EGF (100ng/ml) for the indicated times. ERK1/2 and AKT activation were assessed in cell lysates with specific antibodies. Data are mean +/- SEM of 2-6 independent experiments.* denotes statistical significance between stimulated over-expressing GRK2 and parental 184B5 cells and T between treated and untreated parental 184B5 cells (* or T $p< 0.05$, ** or TT $p< 0.01$ and *** or TTT $p< 0.001$).

7.3. HDAC6 is involved in the GRK2-mediated activation of the EGF pathway.

During the development of this thesis a novel role of the cytoplasmic type II histone deacetylase 6 HDAC6 in sustaining EGFR activation has been published (Deribe et al., 2009). Enhanced HDAC6 activity towards tubulin contributes to reduce the microtubule (MT)-guided intracellular trafficking and to maintain the EGFR at the plasma membrane, thus promoting sustained activation of downstream cascades, which results in enhanced cell proliferation and survival (Deribe et al., 2009; Gao et al., 2010). Since our group recently described a functional interaction between GRK2 and HDAC6 (Lafarga et al., 2012a), we sought to analyze if this new interplay could also contribute to the ability of GRK2 to enhance the activation of signalling cascades downstream growth factor receptors.

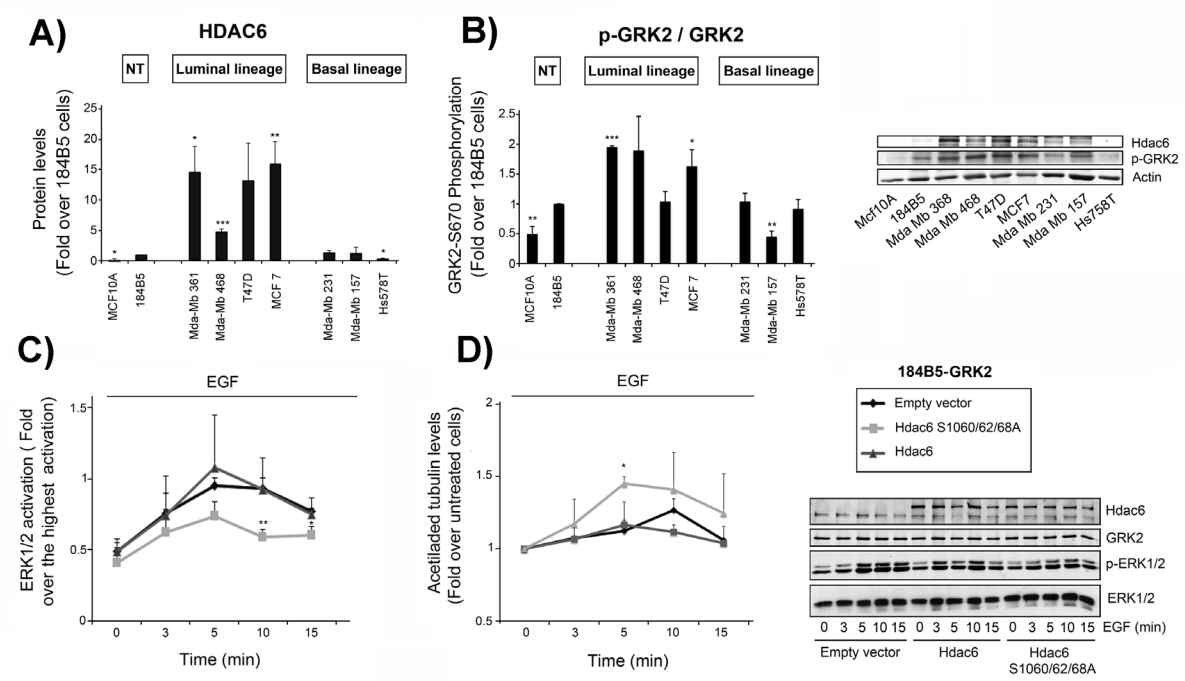


Figure R.19. The GRK2-dependent enhancement of growth factor signalling in breast cells involves GRK2-mediated HDAC6 regulation. A-B) Luminal breast tumour cells display parallel increases pSer670-GRK2 and total HDAC6 protein levels.. Data (mean ± SEM) were expressed as the fold-change over the non-transformed 184B5 cells (n=2-4), with actin serving as loading control. (C-D) GRK2-mediated phosphorylation of HDAC6 underlies the positive effect of GRK2 on EFG-triggered MAPK stimulation. Stably GRK2 over-expressing 184B5 cells were co-transfected with tagged HA-ERK1 and the indicated GFP-HDAC6 constructs. Levels of ERK1 activation (C) and Tubulin acetylation (D) were analyzed as indicated in Material and Methods. Data are mean ± SEM(n=3) *p< 0.05, **p<0.01 and ***p< 0.001 in all panels.

Interestingly, we have recently reported that GRK2 dynamically and directly associates with and phosphorylates HDAC6 to specifically stimulate its alpha-tubulin deacetylase activity at discrete cellular localizations in epithelial cells (Lafarga et al., 2012a). Remarkably, phosphorylation of GRK2 itself at S670 by ERK1/2 was required

for the ability of the kinase to phosphorylate and regulate HDAC6, and GRK2-S670 phosphorylation was promoted by stimuli such as EGF. Notably, enhanced levels of both HDAC6 and S670-phosphorylated GRK2 were detected in all cells in our panel that displayed GRK2 up-regulation (Fig. R.19A and B). Moreover, the increased EGF-mediated signaling to ERK1/2 observed in 184B5 cells stably over-expressing GRK2 was not observed upon transfection of an HDAC6 mutant (HDAC6-S1060/1062/1068A) unable to be phosphorylated by GRK2 and previously shown to block the GRK2-mediated HDAC6 effects on cell motility (Lafarga et al., 2012a) (Fig. R.19C). Consistently, enhanced levels of tubulin acetylation upon EGF treatment were also detected in the presence of the HDAC6 mutant (Fig. R.19D), in agreement with the expected decrease of HDAC6 activity in such conditions. These data strongly suggest that a reinforced GRK2/HDAC6 functional interaction was playing a role in enhancing EGF signalling upon GRK2 up-regulation in breast cells and strongly point to GRK2 as a novel contributor of the cell transformation through the regulation of several substrates such as HDAC6 or Mdm2.

7.4. GRK2 modifies the activation pattern of several transcription factors controlled by p53 upon EGF treatment.

Growth factor dependent signaling is often accompanied by reprogramming of gene-expression pathways that cooperate with the mitogenic networks primary harnessed by deregulated signals/factors in order to trigger aberrant proliferation. Therefore, we analyzed the effect of GRK2 dosage on the activation state of key transcription factors involved in a variety of biological processes, including cell proliferation, differentiation, transformation, and apoptosis by using a profiling assay from Panomics (see Methods) (Fig. R.20A). Upon chronic EGF stimulation, stable GRK2-overexpressing 184B5 cells displayed remarkable changes in the activity of transcription factors (33 of a total of 56 factors analyzed were less active, 5 more active and the remaining factors unaltered, compared to EGF-treated parental cells) (Table2). Interestingly, some over-activated transcription factors in our panel such as AP1 or NFY have been found upregulated in gene-expression meta-analysis of metastasizing breast tumors (Thomassen et al., 2008), and are directly controlled by tyrosine kinase receptors of the ErbB family (Mechta et al., 1997), which is consistent with the positive role of GRK2 in the activation of growth receptor signaling pathways.

Moreover, GRK2 seems to contribute to the over-activation of transcription factors with predominant roles in breast cancer such as CBF, which has been found mutated in breast cancer and may also have a role in aberrant ER signalling in luminal tumours (Banerji et al., 2012); STAT1, which enhances breast tumor growth and immune suppression (Zhang, 2013) or the NF1 family of transcription factors, which has been shown to interact

with hormone receptors, histones, and histone deacetylases (Ray et al., 2013). In addition, transcriptional activity of the up-stimulated Ets-1 factor is known to be enhanced by ERK1/2 (Wasylyk et al., 1997), suggesting a role for GRK2-mediated MAPK stimulation in its over-activation.

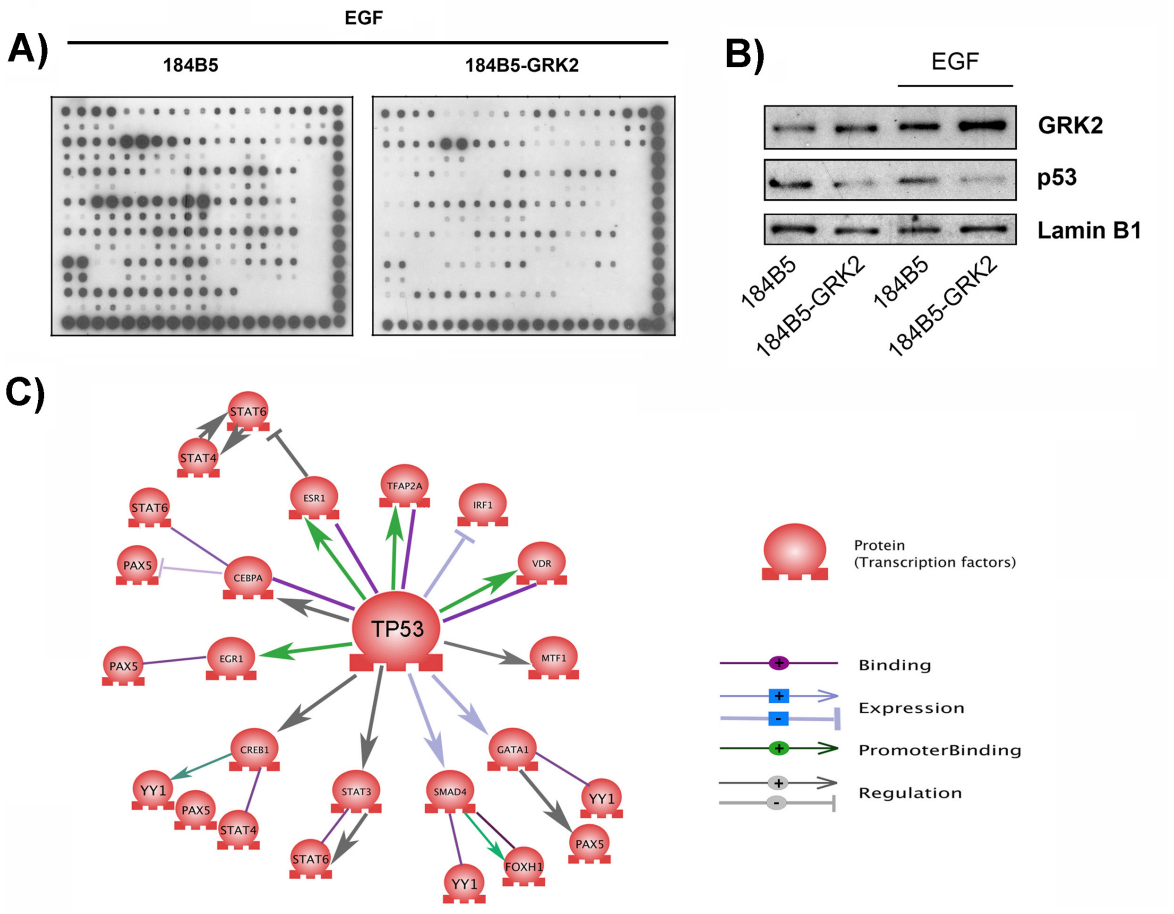


Figure. R.20. Stable over-expression of GRK2 promotes remarkable changes in the activity of several transcription factors upon chronic mitogenic stimulation of non-transformed breast 184B5 cells. A) Parental and stable GRK2 over-expressing 184B5 cells were treated with EGF (100 ng/ml) for 18h and nuclear extracts obtained as indicated in Methods section were incubated with a probe mix containing biotin-labelled DNA binding-consensus sequences for 48 transcription factors included in the TranSignal™ Protein/DNA Array I (Panomics, Redwood City, CA), many of them related to biological processes such as cell proliferation, differentiation, transformation, and apoptosis Representative hybridized array membranes for each experimental condition are shown in panel B. Each spot on the array corresponds to a specific transcription factor. B) Extracts from EGF-stimulated 184B5 cells that stably over-express GRK2 or from parental cells were analysed for the expression of GRK2 and p53 proteins by western blot using laminin B1 as a loading control. A representative gel shows stronger EGF-mediated upregulation of GRK2 levels and down-modulation of p53 in cells with extra GRK2, consisting with the array data of p53 activity. C) Schematic representation of the transcription factors in the array modulated by p53 as determined by Pathway Studio analysis using the Ariadne Genomics database. p53 itself has positive or negative effects on several TFs, which activity is altered accordingly to a p53 downmodulation in cells with extra GRK2.

In contrast, both p53 activity and protein levels were clearly attenuated in GRK2-overexpressing 184B5 cells (Fig.R20B and Table2), in agreement with our previous findings indicating a role for GRK2 in p53 down-modulation. Thus, GRK2 over-expression in 184B5 cells promoted a nuclear decrease in p53 levels both in un-stimulated and EGF-stimulated conditions, whereas EGF treatment promoted an increase in GRK2 protein levels in parental and GRK2-overexpressing 184B5 cells, which strongly supports our previous results in which EGFR-dependent signaling caused up-regulation of GRK2 protein levels in transformed breast cancer cell lines. As a central tumor-suppressive transcription factor, p53 regulates the expression of many genes, including the induction of transcriptional regulators as ER or GATA, which loss is a negative prognostic factor in breast cancer (Troester et al., 2006; Wei et al., 2006). In this context, it is tempting to suggest that the prevailing down-modulation of other transcription factors observed in our panel could be directly or indirectly regulated by the GRK2-mediated control of p53 (Fig. R.20C).

TF	184B5 GRK2	TF	184B5 GRK2	TF	184B5 GRK2	TF	184B5 GRK2	TF	184B5 GRK2	TF	184B5 GRK2	TF	184B5 GRK2	TF	184B5 GRK2
AP-1	n.c	c-myb	-	Ets1/ pea3	+	IRF-1	n.c	NF-E2	-	PPAR	-	Sp-1	-	TFIID	-
AP-2 (TFAP2A)	-	AP-1 (2)	+	FAST-1 (FOXH1)	-	MEF-1	n.c	NFkB	-	PRE	-	SRE	n.c	TR	-
AR	-	CREB	-	GAS/I SRE	+	MEF-2	-	OCT	-	RAR/ DR5	-	Stat-1	-	TR/DR 4	-
BRN-3	-	E2F-1	-	AP-2	-	Myc/M ax	-	P53	-	RXR/ DR-1	-	Stat-3	-	USF-1	-
CEBP	n.c	EGR	n.c	GATA	-	NF-1	+	Pax-5	-	SIE	n.c	Stat-4	-	VDR/ DR-3	-
CBF	+	ER (ESR1)	-	GR/ PR	-	NFAT-1	-	Pbx-1	n.c	Smad/ SBE	-	Stat-5	n.c	HSE	-
CDP	n.c	Ets	-	HNF-4	-	NF-E1 YY1	-	Pit-1	-	SMAD -3/4	-	Stat-6	n.c	MRE (MTF1)	-

Table R.2. Table summarizing changes in the activity of TFs expressed in stable GRK2 over-expressing 184B5 cells compared to that of parental cells. Upon chemoluminescence detection, the signal intensity of spots were subjected to scanning densitometry and normalized to control positive probes. The transcription factors whose activities were increased or decreased more than 1.5 fold compared to parental cells were taken as over-activated (+; red colour) or down-activated (-; blue colour), whereas variations of less than 1.5 fold were defined as no changes (n.c.).

7.5. The increase of GRK2 levels in non-transformed breast cancer cell lines confers growth and survival advantages under stress conditions.

During tumorigenic progression, cancer cells continuously encounter various growth-constraining conditions, such as low oxygen pressure (i.e., hypoxia), lack of cell-cell contact, insufficient support by the extracellular matrix (possibly leading to anoikis)

or nutritional deprivation. Thus, the cellular adaptive response to environmental stresses is recognized as an important mechanism that facilitates tumorigenic progression. Under stress conditions, cancer cells may secrete many autocrine and/or paracrine factors that eventually induce a favorable environment for tumor growth. These signals usually converge on the PI3K-Akt pathway to support cell growth and survival (Bruno et al., 2007; Gao et al., 2014).

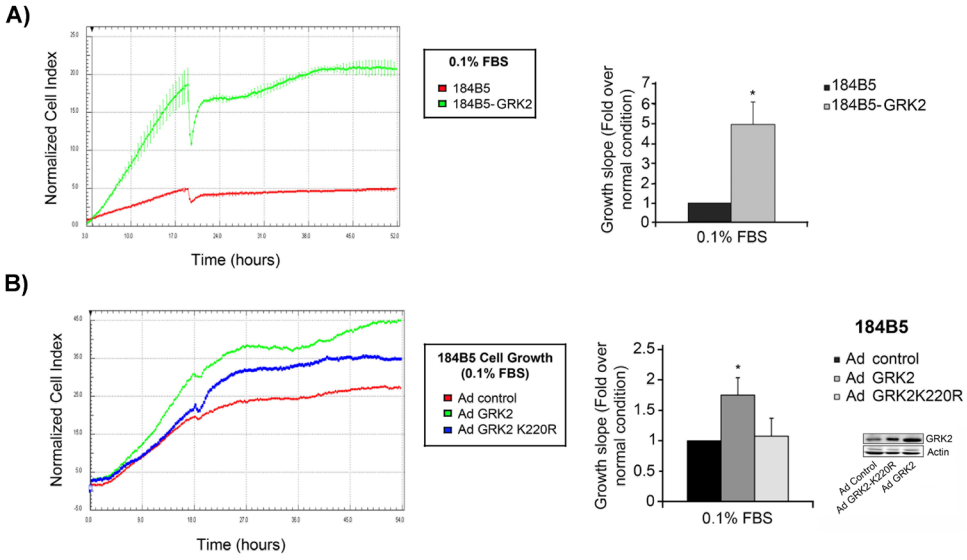


Figure R.21: (A-B) The kinase activity of GRK2 fosters cell proliferation of non-transformed cells under restrictive growth conditions. Growth in 0.1% FBS of parental, stably GRK2 over-expressing (A) or transduced with the indicated GRK2 constructs (B) 184B5 cells was monitored with the xCELLigence technology as described in Materials and Methods. Growth slope data (mean ± SD) are from 2 (A) or 4 (B) independent experiments performed in duplicate or triplicate. *p<0.05, **p<0.01, when compared to parental or control conditions.

Based on our results indicating that GRK2 levels enhance signaling through the PI3K/Akt and Ras pathways and supports apoptosis resistance upon genotoxic treatment, we next investigated whether increased GRK2 levels could endow normal breast cells with proliferative advantages in adverse conditions. Serum starvation is a physiologically relevant growth-constraining condition that provides an experimental system to analyze how normal cells undergo apoptosis in response to nutrients deficiency, and how malignant cancer cells successfully adapt and can continue their malignant growth (Mahbub Hasan et al., 2012). We found that stable over-expression of GRK2 in 184B5 cells promoted a 5-fold increase in proliferation rate in low-serum conditions compared to parental cells, as monitored in a real time, cellular impedance-based growth assay (see Methods) (Fig. R.21A). Consistent with a relevant role for GRK2 kinase activity in the control of cell proliferation, adenoviral-mediated overexpression of the catalytically inactive GRK2-K220R mutant in 184B5 cells was unable to promote cell proliferation in these conditions as the wild-type kinase did (Fig. R.21B).

8.

GRK2 levels and activity modulate cell growth of transformed breast cancer cell lines

8.1. GRK2 activity modulates proliferation of luminal-like breast cancer cells independent of their p53 status.

Overall, our data indicated that up-regulation of GRK2 levels in untransformed breast cells modulated, in a way dependent on its kinase activity, integrated cellular responses such as growth or survival. We next investigated whether altering GRK2 levels in transformed breast cancer cells that already displayed high kinase levels also regulated cell proliferation. In the luminal cancer cell line MCF7, which retains wild type p53, further increasing GRK2 dosage by means of an adenovirus construct strongly fostered the already high growth rate of these cells in normal serum conditions, whereas the presence of a catalytically inactive mutant of GRK2 (K220R) had no comparable effect (Fig. R.22A). Furthermore, as shown in figure R.22B, GRK2 also promotes an upward trend in cell proliferation in the p53-mutated (p53-R273H) MDA-MB-468 cells whereas silencing its expression had a strong negative effect. Such negative effect in cell proliferation was not rescued with the catalytically inactive GRK2 mutant, indicating again a kinase-dependent

mechanism. These results suggested that GRK2 plays a relevant role in the growth of cells harbouring p53 mutations. This finding raised the question of a possible role of GRK2 in "canonical" basal breast cancer cells (in which 80% present mutations in p53), despite that fact that kinase levels were not increased, in contrast to transformed cells of luminal lineage.

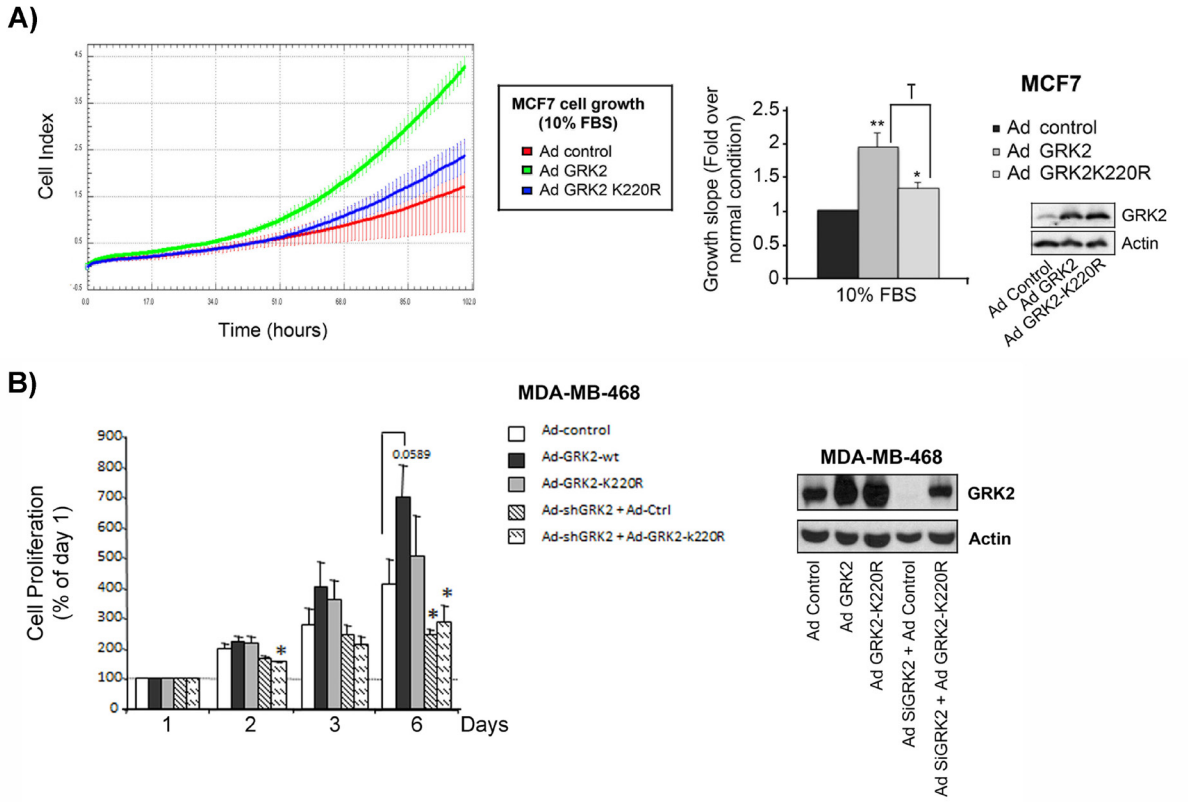


Figure R.22: GRK2 modulates cell growth of luminal transformed breast cancer cell lines. A) Luminal MCF7 cells were infected with different adenoviral constructs of GRK2 (either wild type, the kinase-dead mutant K220R or with a control adenovirus as indicated). Cells growing in 10% FBS-supplemented culture medium were seeded either onto 96-well gold electrode sensor plates to monitor changes in cell index for 4-5 days. Data (cell growth slope) are the mean ± SEM of 3 independent experiments performed in duplicate. p values were * or T $p<0.05$ and ** $p<0.01$ when compared between conditions or to control infected cells. Representative cell index graph and western blot of transduced GRK2 protein levels are shown . B). MDA-MB-468 cells were infected with a control adenovirus or different adenoviral constructs of GRK2 (wild-type, shGRK2, GRK2-K220R or a combination of shGRK2 and GRK2-K220R). 48 hours after infection, cells were seeded onto a 96-well plate and cell growth ws measured using a MTT proliferation assay. Data (mean +/- SEM of 3 independent experiments) are represented as the percentage of growth over the 24 hours proliferation state. (*$p< 0.05$)

8.2. GRK2 controls cell proliferation of the basal-like transformed breast cancer MDA-MB-231 cell line through the modulation of HDAC6.

Recent evidences indicate that the p53-R280K mutation, present in the triple-negative breast cancer cell line MDA-MB-231, acts as a gain of function p53 mutant and has an important role in mediating the cell growth and survival of these cells (Bae et al., 2013). Therefore, we choose this cell line in order to establish the effect of modulating

GRK2 levels in cell growth of a "canonical" basal breast cancer cell. As shown in Figure R.23A, knockdown of GRK2 levels clearly inhibited MDA-MB-231 cell growth, whereas the induction of extra GRK2 by infection with adenovirus promoted a slight increase in cell proliferation. Moreover, the effect of GRK2 on cell proliferation of MDA-MB-231 cells would require the kinase activity of GRK2, since the over-expression of the catalytically inactive form of GRK2 cannot increase cell proliferation as efficiently as the wild-type form of GRK2 (Fig.R.23B). These data strongly suggested that GRK2 could also play a role in the tumorigenesis of basal breast tumours harbouring p53 mutations.

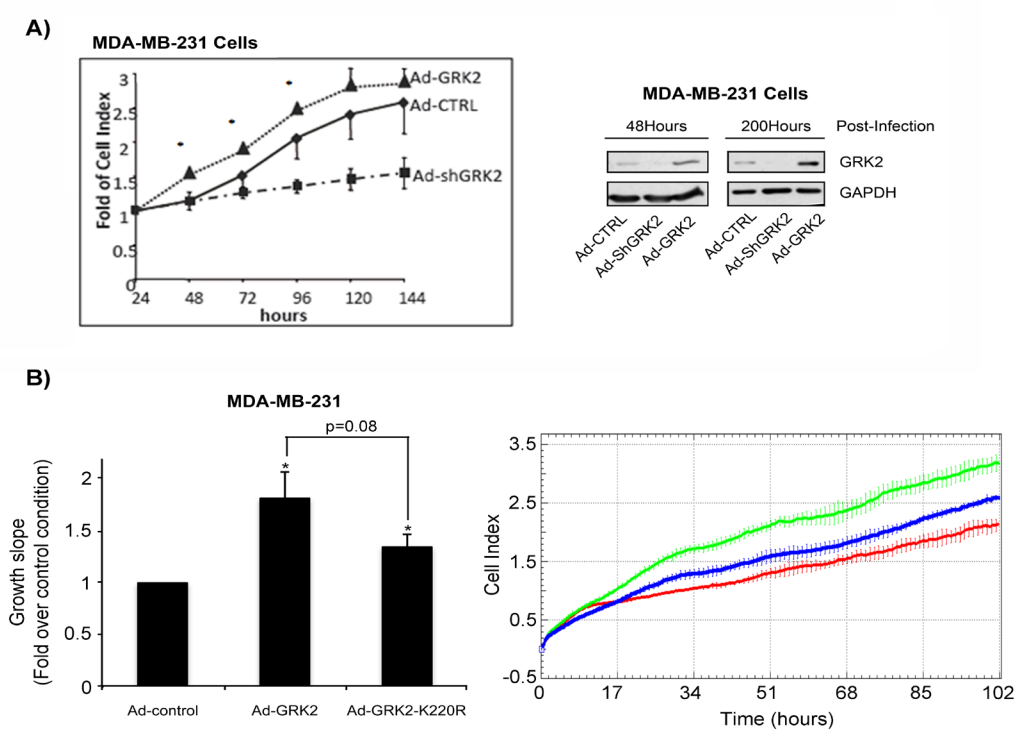


Figure R.23: GRK2 also modulates cell growth of "canonical" basal transformed breast cancer cell lines. A) MDA-MB-231 cells were infected with adenoviral construcs of GRK2 (wild type or ShGRK2) or with a control adenovirus and cell growth was assessed using the XCellLigence System. At indicated times, fold of the cell index (± SEM) from 3 independent experiments are shown. GRK2 expression levels at the the beginning and at the end of the experiment were measured by western blot to ensure the proper functioning of the adenoviral infection. B) MDA-MB-231 cells were infected with adenoviral construcs of GRK2 (wild type or GRK2-K220R) or with a control adenovirus and cell growth was assessed using the XCellLigence System. Cell growth slope was calculated as in Fig. R.21. Data (mean ± SEM of 3 independent experimental approaches) are represented.

To investigate whether the GRK2-dependent phosphorylation of HDAC6 was involved, we generated MDA-MB-231 breast cancer cells engineered using the Gateway system to stably over-express wild-type HDAC6 or the GRK2-phosphorylation defective mutant HDAC6-S1060/1062/1068A. Remarkably, expression of wt-HDAC6 strongly increased the proliferation slope of MDA-MB-231 cells, whereas expression of the HDAC6S1060/1062/1068A mutant enhanced tubulin acetylation status and did not mimic the effect of wt HDAC6 on cell proliferation (Fig. R.24A-B).

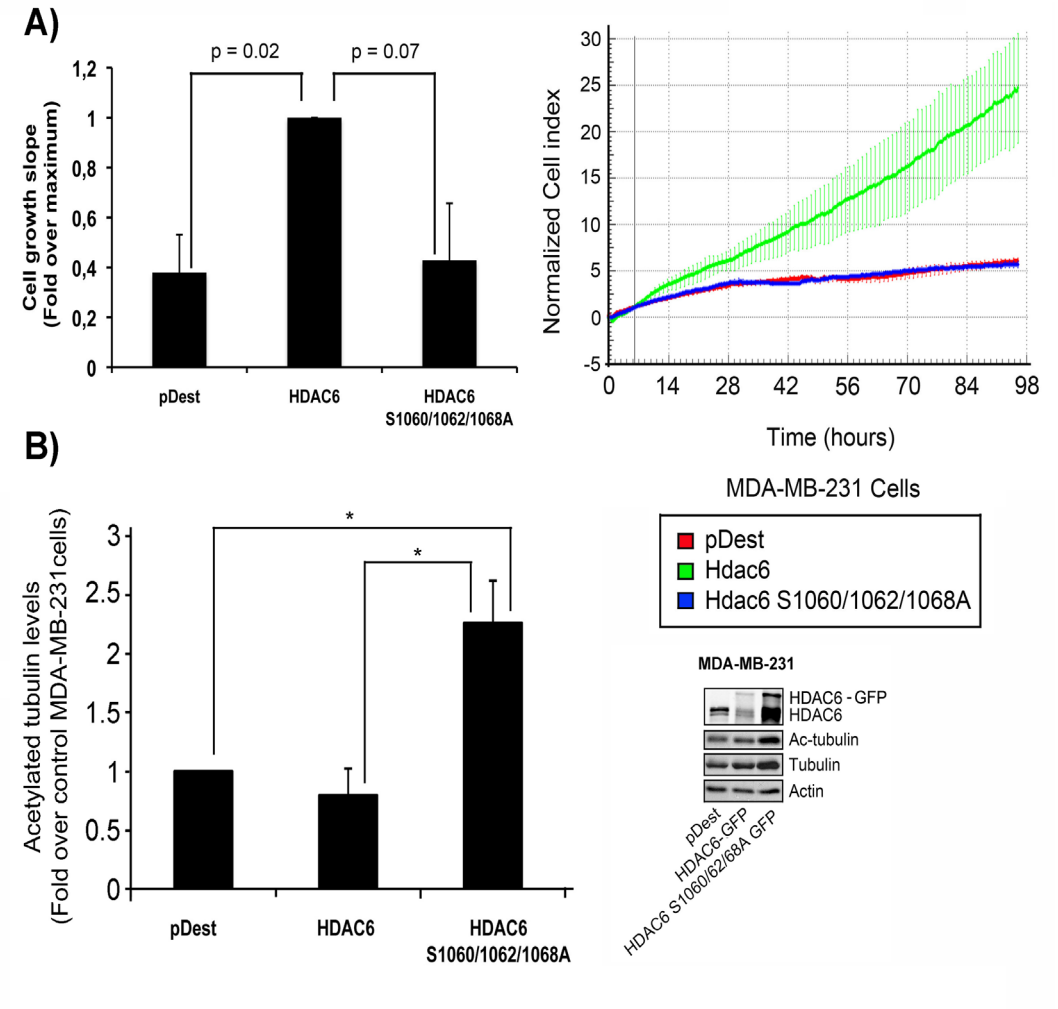


Figure R.24: GRK2-dependent phosphorylation of HDAC6 is essential for cell growth of MDA-MB-231 cells. A) Stably transfected MDA-MB-231 cells over-expressing GFP-HDAC6-wild type or the phosphodefective HDAC6 mutant GFP-HDAC6 S1060/1062/1068A were seeded onto a 96-well gold electrode sensor plate in 10% FBS and cell growth was monitored with the xCELLigence technology. Slope data is expressed as mean ± SD of 2 independent experiments and normalized over the maximum cell slope (*$p<0.05$). A representative experiment is shown. B) Cells were collected at the end of the experiment and lysed to analyzed the levels of HDAC6 expression and actetylated tubulin by western blot. Acetylation levels of tubulin were normalized by the total levels of Tubulin. A representative western blot and Data (mean ± SD of 2 independent experiments performed by triplicated) are shown.

9.

Subcellular distribution of GRK2 is disturbed in p53-mutated breast cancer cell lines

Since we could not detect any changes in total GRK2 expression in the basal lineage cells analyzed (Fig R.1), we searched for changes in normal kinase subcellular distribution that could support our new finding. Recent publications point that GRK2 is localized to centrosomes and plays a central role in mediating EGFR–promoted separation of duplicated centrosomes (So et al., 2013). These findings pose new questions regarding the potential role of GRK2 in the control of nuclear features. Thus, we investigated whether GRK2 changes its sub-cellular distribution in cell lines harbouring mutations in p53 such as MDA-MB468 (p53-R273H), T47D (p53-F194L), MDA-MB-231 (p53-R280K) and Hs578T (p53-V157F). As shown in Figure R.25A, all p53-mutated breast cancer cell lines displayed a notable increase of GRK2 in the nuclear fraction, compared to the wild-type p53 and non-transformed breast cell lines MCF10A and 184B5. This effect seems to be independent of the breast cancer subtype, since MDA-MB-468 and T47D present a more luminal phenotype whereas MDA-MB-231 and Hs578T cells belong to the "canonical" basal group. Interestingly, a marked up-regulation of GRK2 phosphorylation at S670 was

specifically noted in parallel in the nuclear fraction of GRK2 (Fig. R.25B), suggesting that this post-transcriptional modification of GRK2 would confer a novel layer of regulation by switching the sub-cellular distribution of the kinase. Of note, as mentioned above, GRK2 is robustly phosphorylated at S670 in response to EGF (Penela et al., 2008), and such modification is instrumental in enhancing localized phosphorylation of HDAC6 in situ (Lafarga et al., 2012a). Thus, it is tempting to suggest that phosphorylation of GRK2 at S670 acts as a signalling switch to potentiate cell proliferation and survival responses. However, the precise molecular mechanisms by which GRK2 is phosphorylated during breast cancer transformation and the possible nuclear function of GRK2 remain to be elucidated.

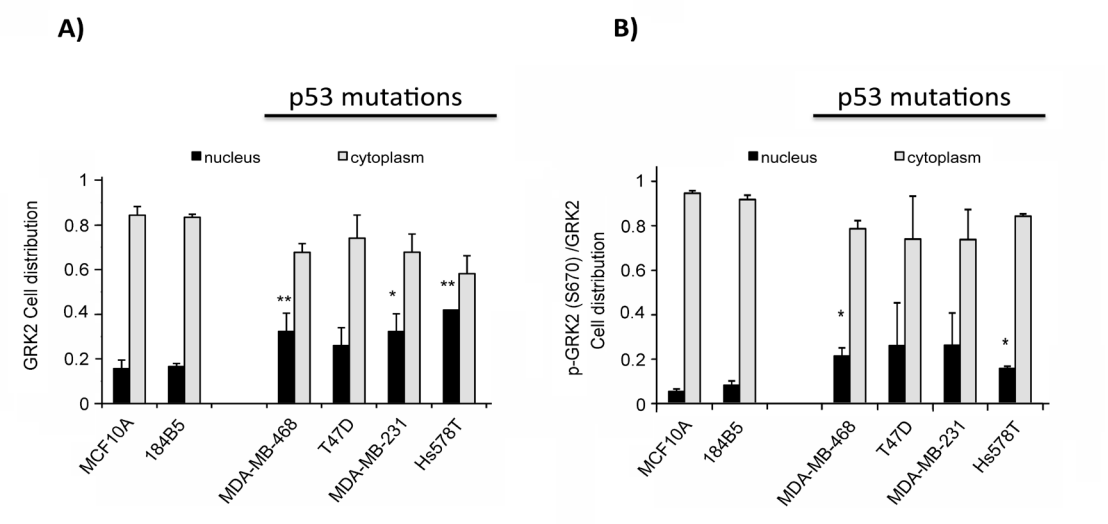


Figure R.25: Sub-cellular distribution of GRK2 is disturbed in p53-mutated breast cancer cell lines. Non-transformed breast cell lines and different breast tumour cells expressing mutant p53 were subcellularly fractionated. The protein levels of GRK2 (A) , and GRK2-phospho-Serine 670 (B) were analyzed in cytoplasmic and nuclear fractions with specifics antibodies. Nucleolin and GAPDH content was used to monitor the accuracy of cellular fractionation and as loading controls. Data (mean ± SEM) from 3 independent experiments are represented as the protein expression distribution between the nuclear and cytoplasmic fraction expressed as %. (*p< 0.05 and **p< 0.01).

10.

GRK2 promotes cellular resistance to chemotherapeutic drugs

Resistance to chemotherapy is another important feature of cancer progression. As shown in previous figures, enhanced GRK2 levels conferred resistance to apoptosis triggered by the commonly used genotoxic compounds paclitaxel, etoposide or doxorubicin in wild-type p53 harbouring cells. This effect seemed to rely on the GRK2-dependent control of the Mdm2/p53 regulatory axis. However, mutations of p53 protein occurs in more than 20% of breast human cancers, and mutant p53 display protein hyper-stabilization turning to ineffective those therapies based in the disruption of p53/Mdm2 interface. In this regard, it has been recently reported that the interaction of the chaperone Hsp90 with mutant p53 stabilizes its conformation and blocks its degradation by inhibiting Mdm2 activity (Li et al., 2011b, 2011a). In addition, the activity of HDAC6 has been involved in the stabilization of mutant p53 in MDA-MB-231 cells by means of maintaining Hsp90 in an active state (Kovacs et al., 2005; Li et al., 2011b). Therefore, we sought to determine whether the interplay of GRK2 with HDAC6 and Mdm2 might have consequences in the response of wild type and mutant p53 to different chemotherapeutic drugs.

10.1. GRK2 protects MCF7 breast cancer cells from Nutlin3a-induced death in a kinase dependent manner

To assess the potential relevance of the GRK2/Mdm2 pathway in this context, we utilized the wild-type p53 transformed breast cancer cell line MCF7, where over-expression of GRK2 decreased the Nutlin3a-induced p53 response in a kinase dependent manner. We compared cell growth arrest by different dosages of Nutlin 3a in control, GRK2 and GRK2-K220R populations (Fig. R.26). Nutlin-3a inhibited cell growth in all three populations in a dosage dependent manner. However, the presence of extra GRK2 markedly decreased growth arrest at low Nutlin3a doses. This effect seems to be kinase dependent since extra GRK2-K220R did not alter the Nutlin3a outcome.

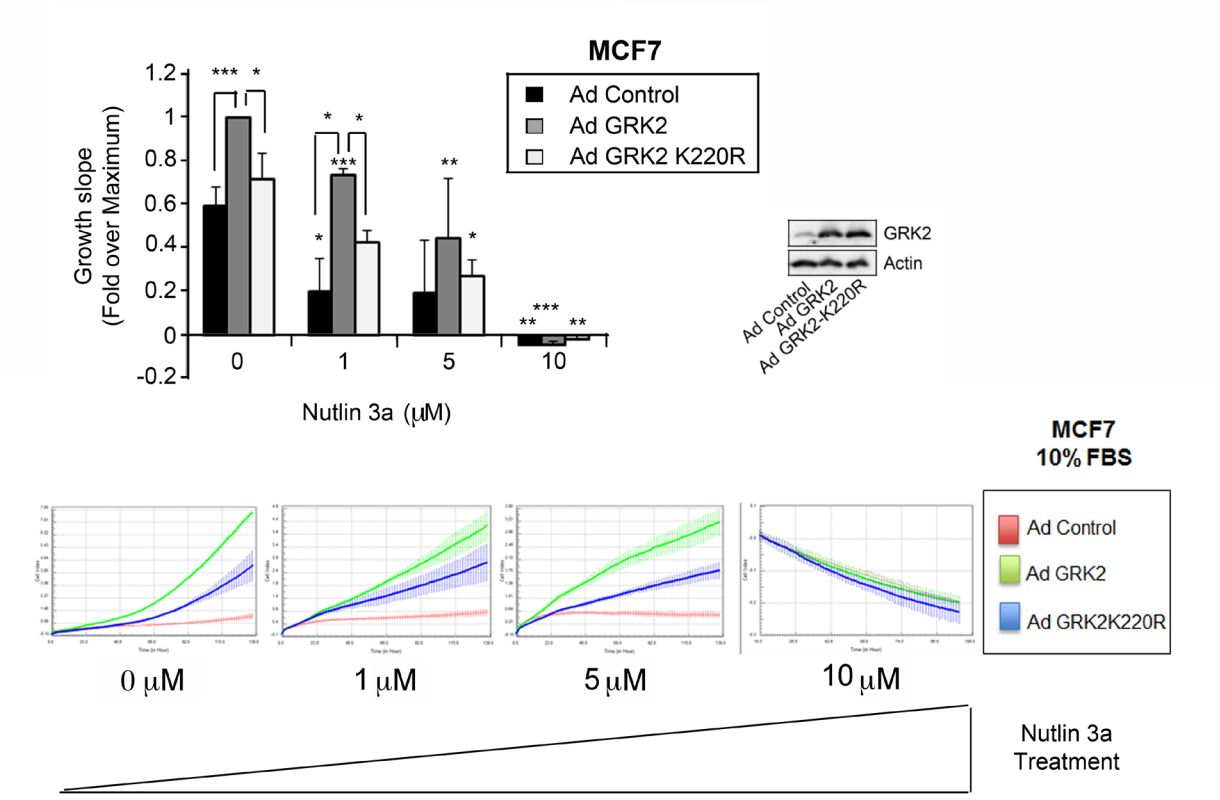


Figure R.26: GRK2 protects from Nutlin3a-induced cell death of MCF7 cells in a kinase-dependent manner. MCF7 cells transduced with different adenoviral constructs (control, GRK2 and GRK2-K220R) were pretreated for 2 hours with indicated concentrations of Nutlin3a and (10000 cells) and seeded into 96-well E-plates in the presence of 10% FBS. Cellular impedance was continuously recorded and converted to a cell index (CI) for cell proliferation determination. CI values were normalized after the adhesion stage. The slope of the resulting growing curves are plotted. Data are mean ± SEM of 2-4 independent experiments performed in duplicate. (*p< 0.05, **p< 0.01 and ***p< 0.001). Representative growth curves for increased Nutlin3a dosages are shown.

10.2. GRK2 decreases SAHA and tubacin-induced death in non-transformed breast cancer cell lines (184B5 cell line) in a kinase dependent manner

Emerging evidence indicate that HDAC6 inhibitors can also be used as anti-cancer agents. Pan-HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA), the first and currently only FDA-approved, or tubacin, a specific HDAC6 inhibitor that binds only to the tubulin deacetylation domain, are able to induce growth inhibition and apoptosis in different cancer cell types (Marks & Breslow, 2007; Marks & Jiang, 2005; Namdar et al., 2010). Since enhanced GRK2 levels would potentiate HDAC6 activity in the wild type p53, non-transformed breast cell line 184B5, we reasoned that the anti-proliferative and death-promoting effects of these HDAC6 blockers would be attenuated upon GRK2 upregulation. Cell growth of 184B5 cells in serum-deprivation conditions usually occurs during the first 40-50 hours. Afterwards, cells show growth arrest that finally ends in cell death (Fig. R27A) We have monitored both events, defining the first period as "cell proliferation", and the second one (from 40 to 100-150 hours) as "cell death", in order to determine if altering GRK2 levels modulates cell proliferation and/or or can revert cell death by increasing cell survival. Interestingly, incubation of 184B5 cells with SAHA or tubacin caused a strong inhibition of proliferation and induction of cell death in low serum conditions, and these effects were markedly counteracted upon adenovius-mediated overexpression of wild-type (but not the kinase-inactive K220R mutant) GRK2 (Fig. R.27B), further suggesting that high levels of this protein confers survival properties to breast cells in the presence of a variety of apoptosis inducers by mechanisms involving its kinase activity.

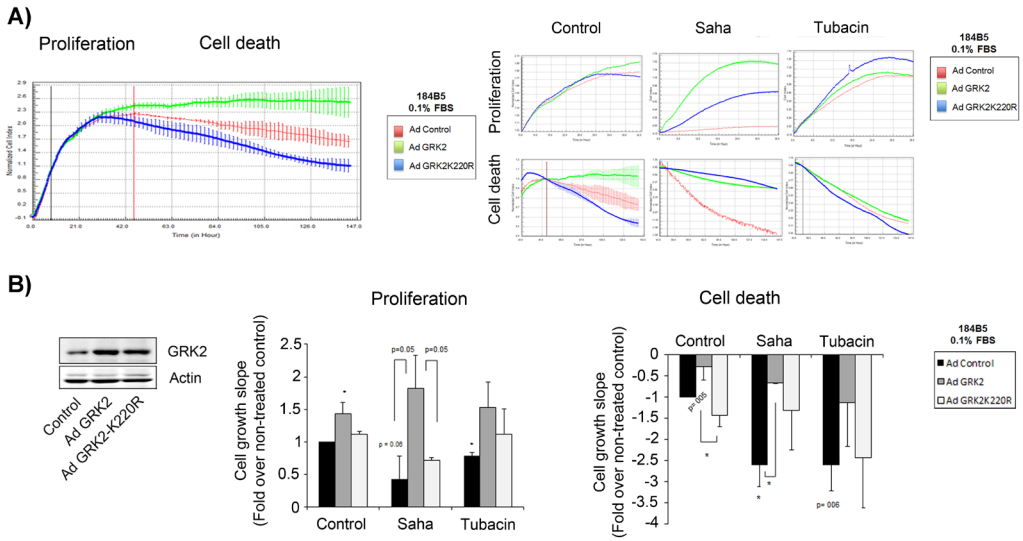


Figure. R.27: GRK2 kinase activity attenuates the pro-arresting and pro-death effects of HDAC inhibitors in non-transformed cells Pro-arresting and pro-death effects of the pan-HDAC inhibitor SAHA or tof he specific HDAC6 inhibitor tubacin were monitored using the xCellLigence System in 0.1% FBS-growing 184B5 cells transduced with wt GRK2 or mutant GRK2-K220R as described in Methods. Data of cell index slopes represent mean ±SEM of n=2-4 experiments (*p<0.05).

10.3. GRK2 confers survival properties upon SAHA treatment in a p53-mutated context

Recent evidences reveal that SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-HSP90 chaperone axis (Li et al., 2011a). We thus explored whether the positive regulation of HDAC6 by GRK2 could modulate the pharmacological responses of MDA-MB231 cells to HDAC6 inhibitors Therefore, we compared the effect of SAHA on the viability of MDA-MB231 cells that over-express wild type GRK2 to that on parental cells. Increased GRK2 levels enhanced normal growth of MDA-MB-231 cells (Fig. R.28A), clearly and significantly attenuated SAHA-induced cytotoxicity (Fig. R.28B) and markedly increased the IC50 of the drug (circa2-fold) (Fig. R.28C).

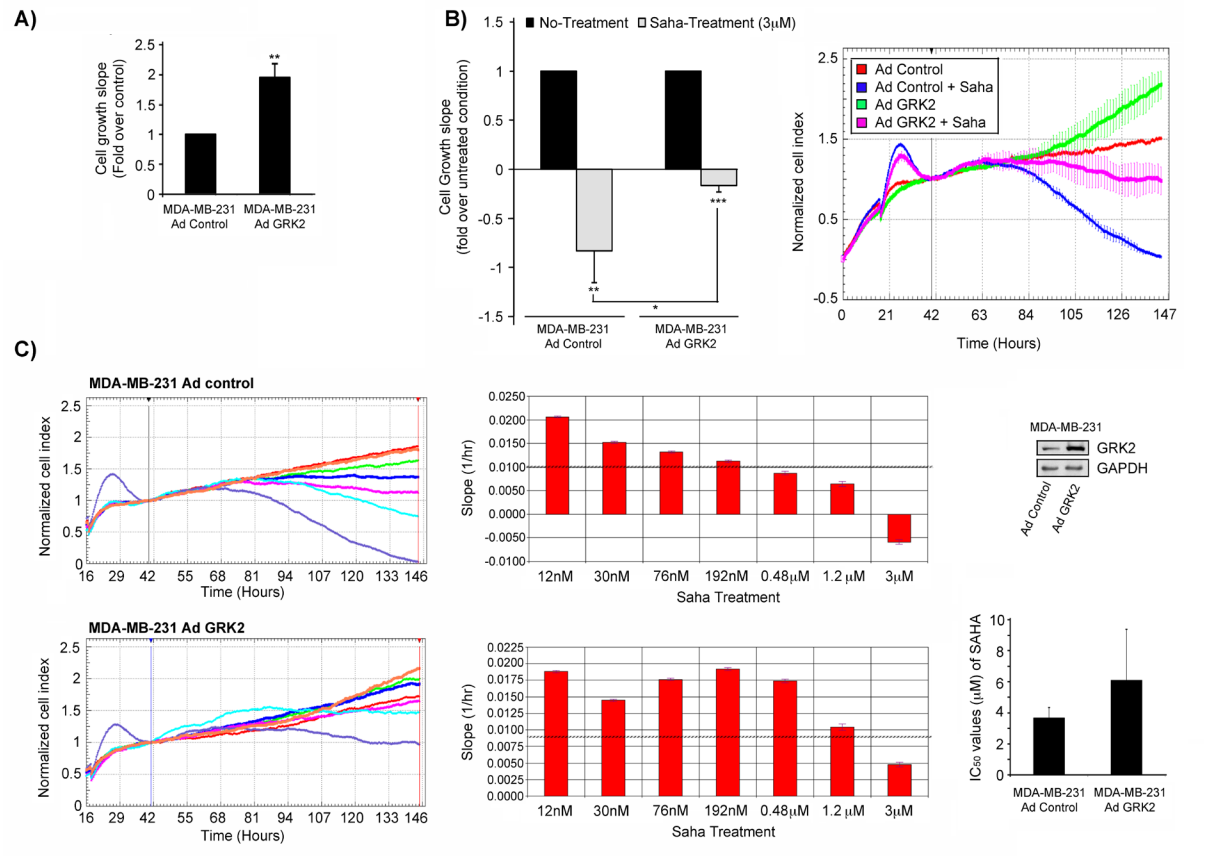


Figure R.28: GRK2 lessens SAHA-induced death in breast cancer cells with mutant p53. A-B) Pro-arresting and pro-death effects of the pan-HDAC inhibitor SAHA were monitored using the xCellLigence System in 10% FBS-growing MDA-MB-231 cells transduced with wt GRK2 or a control adenovirus. Data of cell index slopes at 3μM of Saha treatment represent mean ±SEM of n=4 (*p<0.05; **p<0.01; ***p<0.001). C). MDA-MB-231 cells infected with GRK2 or adenovirus control were seeded onto a 96well plate of XCellLigence. After 24 hours, SAHA compound was added at the indicated increasing concentrations. Cell index values were normalized after SAHA addition. Cell growth slopes are represented and IC50 was calculated using the software of the XCellLigence as described in Materials and Methods section. Data are mean ± SD of 2 independent experiments performed by duplicate or triplicate.

11.

GRK2 favours the anchorage-independent growth of breast cancer cells in a kinase activity-dependent manner

Resistance to cell-detachment-induced apoptosis (also termed anoikis) allowing anchorage-independent growth is one of the hallmarks of cell transformation (Eckert et al., 2004) and is related to increased aggressiveness (Guadamillas et al., 2011; Hanahan & Weinberg, 2011). In this process, malignant cells are able to survive and proliferate without cell adhesion and junctions–dependent signaling (Lee et al., 2008). Key molecular mechanisms underlying this tumoural hallmark involve PI3K/AKT and Ras-induced override of adhesion requirements for cell cycle progression (Thullberg et al., 2007), and avoidance of anoikis by means of p53 down-modulation (Grossmann, 2002). Because both Ras and p53 functionality are influenced by GRK2 dosage, we investigated whether kinase levels and activity would modulate the capacity of cells to grow in soft agar in MCF7 breast cancer cells engineered to timely induce the expression of wild-type GRK2 or its catalytically inactive mutant GRK2-K220R in the presence of tetracycline (Fig. R.29A). Remarkably, induction of the expression of wt GRK2 strongly increased

(more than 10-fold after 3-weeks in culture) the ability of MCF7 cells to form colonies in soft agar conditions, whereas expression of the GRK2-K220R mutant did not mimic such effect (Fig. 29B-C). Moreover, the proportion of larger colonies (Fig. R.29D) and the overall size of these large colonies (Fig. R.29E) also increased in wild-type versus GRK2-K220R-expressing cells at both 1 week and 3 weeks of culture (Fig. R.29C), what might be ascribed to their own differences in proliferation rates (Fig. R.22). These results indicated that GRK2 kinase activity is a strong facilitator of the processes triggered to subvert adhesion control and to bypass anoikis.

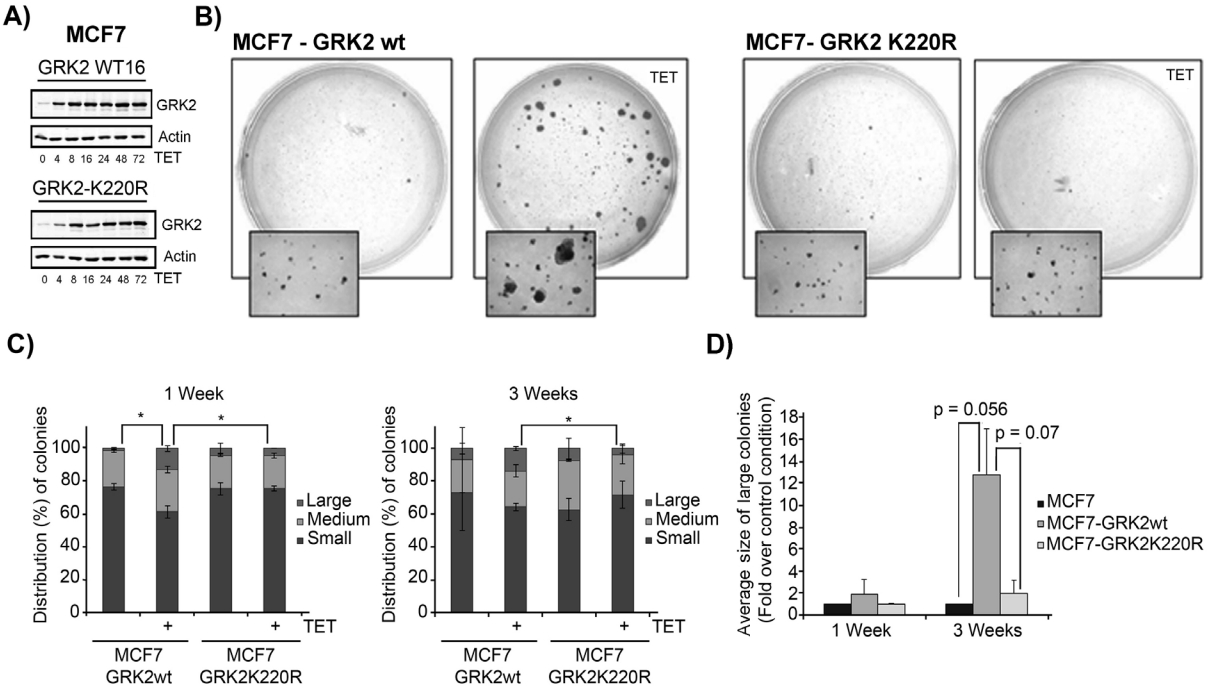


Figure R.29. GRK2 favours the anchorage-independent growth of luminal breast cancer cells in a kinase-dependent manner. (A) Time-course of GRK2 levels in Tet-on wt-GRK2 or mutant GRK2-K220R MCF7 cells treated with tetracycline (TET). (B) Colony formation by stable Tet-on wt-GRK2 or mutant K220R-MCF7 cells in soft-agar medium in the presence or absence of tetracycline analysed as described in Methods section. (C-D) Extra levels of wt-GRK2, but not of inactive kinase, increase both the size and the proportion of large colonies grown in un-anchorage conditions. The area of colonies was measured using Image J and the size of colonies was scored as Large, Medium and Small as described in Methods. Bar-graphs showed the distribution (%) of the colonies according to their size (C) and the median size (D) of colonies. Data are mean ±SD of 2 independent experiments by duplicate. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) .

We carried out a similar experimental approach in a p53-mutated context, using the low GRK2-expressing basal MDA-MB-231 cells. As shown in figure R.30, adenovirus-mediated infection of MDA-MB-231 cells with wild-type GRK2 potentiated the ability of these cells to form colonies in agar, and significantly increased the proportion of larger colonies. More important, downregulation of GRK2 expression by adenoviral delivery of a silencing construct (Ad-shGRK2) completely abrogated the anchorage-independent growth of these cells, suggesting that GRK2 is essential to efficiently escape anoikis and a key contributor to cell growth in these experimental conditions. Moreover, we can conclude that the role of GRK2 in this process is independent of the control of the wt-p53 pathway, since GRK2 also potentiates the anchorage-independent growth in cells that harbour mutations on p53.

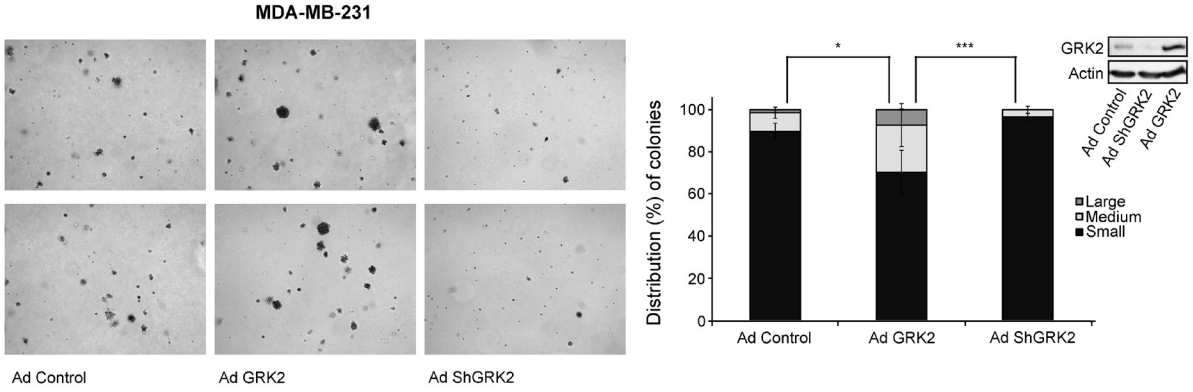


Figure R.30: GRK2 favours the anchorage-independent growth of mutant p53 breast cancer cells in a kinase-dependent manner. Colony formation by MDA-MB-231 cells infected with different adenoviral construcs (Control, GRK2 wild-type and shGRK2) in soft-agar medium as described in Materials and Methods and Fig.R.29. Bar-graphs showed the distribution (%) of the colonies according to their size. Data are mean ±SD of 2 independent experiments performed by duplicate. (* $p<0.05$, *** $p<0.001$) . Lysates of MDA-MB-231 after 48 hours of infection were analyzed by immunoblotting with specific anti-GRK2 antibody to verify the efficiency of the infection.

12.

GRK2 is a relevant modulator of tumour growth in vivo

The different effects of GRK2 described in the sections above strongly supported an important role for GRK2 in promoting oncogenic phenotypes. In order to explore the impact of GRK2 levels in driving and/or maintaining tumor development in vivo, we investigated whether xenograft tumour growth in mice was influenced by the extent of GRK2 expression in human breast cancer cells displaying different molecular signatures in terms of GRK2 expression, p53 status and/or PI3K mutations.

12.1 GRK2 influences in vivo tumour growth promoted by wt-p53 luminal breast cancer cells in a kinase-dependent manner by regulating proliferative and apoptotic pathways.

In a first approach, GRK2 or GRK2-K220R expression was induced by tetracycline in engineered MCF7-Tet-On cells prior to subcutaneous implantation in doxycycline-treated nude mice, and subsequent evolution of tumours was compared. Non-induced MCF7 cells injected in non-doxycycline treated mice were used as controls. We observed that tumours formed by MCF7 cells over-expressing wild-type GRK2 developed earlier and reached significantly higher sizes (Fig. R.31A), whereas no apparent difference was

detected between tumours grown from control or GRK2-K220R-expressing cells (Fig R.31B). Staining of tumour sections demonstrated an increase in labelling of the proliferation marker Ki67 in MCF7-wtGRK2-induced tumours, along with a decrease in p53 and cleaved caspase 3 immunostaining compared to control cells, indicative of resistance to apoptosis, whereas no significant changes with respect to controls were observed in MCF7-K220R mutant-derived tumours (Fig. R31C and D), despite similar expression levels of GRK2 protein were attained. Moreover, preliminary data showed a positive correlation between Mdm2 and GRK2 protein expression in all tumors (Fig. R.31E). Interestingly however, Mdm2 expression was higher in MCF7-wtGRK2-induced tumours than in MCF7-K220R mutant-derived tumours. Overall, these results are consistent with the idea that GRK2 , in a kinase dependent manner, is required for modulation of cell proliferation and survival through the control of p53/Mdm2 axis.

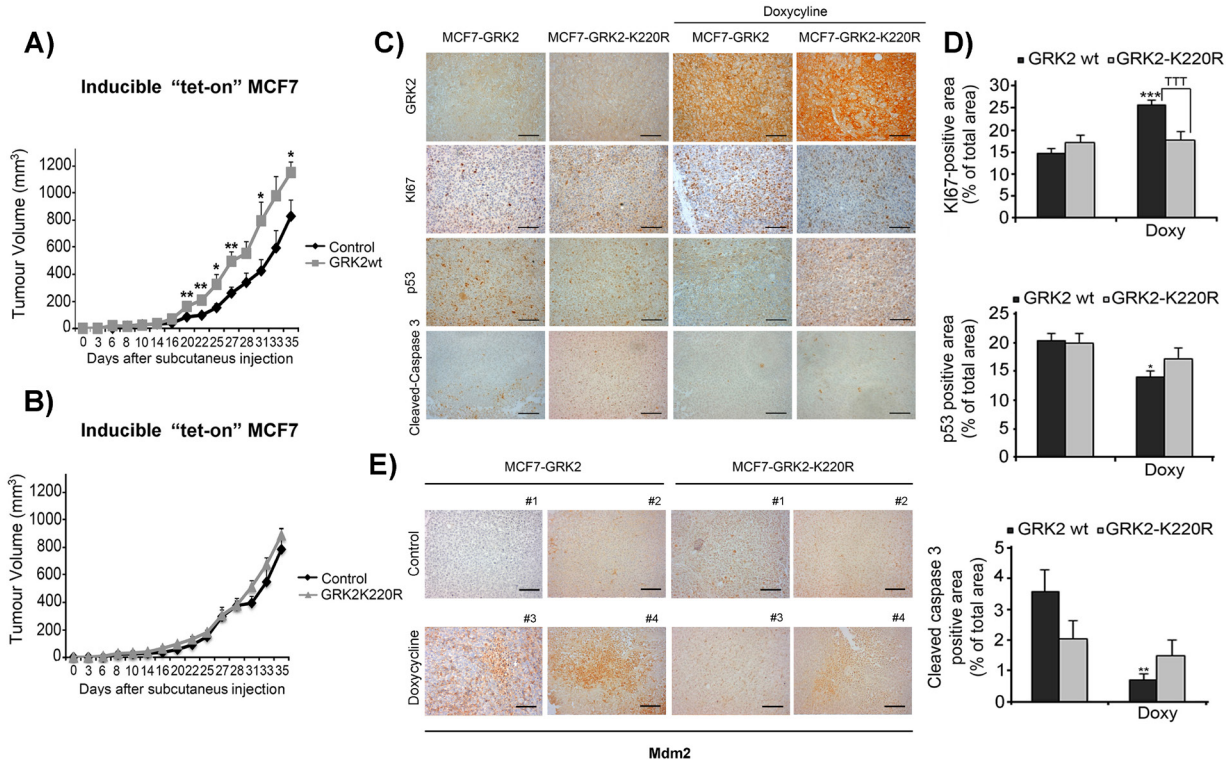


Figure 31. GRK2 modulates *"in vivo"* tumour growth in a kinase-dependent manner by regulating proliferative and apoptotic pathways. (A-B) Tumours formed by MCF7 cells over-expressing wtGRK2, but not the K220R mutant, developed earlier and reached significantly higher sizes. MCF7-TET-ON wt-GRK2 or GRK2-K220R-expressing cells pre-treated with tetracycline or vehicle (control) were subcutaneously implanted in doxycicline-treated or un-treated (control) nude mice. Tumour volume was measured each 2-3 days. Data are the mean ± SEM from tumour masses of 6-8 mice/group (* $p<0.05$, ** $p<0.01$ compared to untreated MCF7-Tet-on cells). C-D) Tumours were removed 30-35 days post-injection and sections analysed with specific antibodies for GRK2, Ki67, p53 and cleaved caspase-3 as described in Methods section. (Scale bar: 100 µm, * $p<0.05$, $p< 0.01$, *** $p<0.001$). E) Immunohistochemical detection of Mdm2 in tumours removed at 30-35 days. MDM2-positive immunoreactivity was found in doxycycline-treated mouse, specially in wtGRK2-derived tumours (Scale bar: 100 µm).

These findings were confirmed using an alternative experimental setting, using adenoviral vectors to increase expression of wtGRK2 or GRK2-K220R in MCF7 cells prior to subcutaneous injection in mice. Again, enhanced wtGRK2 levels stimulated the rate and extent of tumour formation, while control and mutant GRK2 expressing cells behaved similarly (Fig. R.32A). More important, downregulation of GRK2 expression by adenoviral delivery of a silencing construct (Ad-shGRK2) completely abrogated tumour growth, in line with the effect of GRK2 downmodulation on cultured MCF7 cell proliferation and apoptosis induction (see Figs. R.10, R.22 and R.25). Opposite to the effect observed in tumours over-expressing wt GRK2, sections from the small shGRK2-MCF7- derived tumours at early time points indicated that along GRK2 downmodulation a substantial reduction in the proliferation marker Ki67 was taking place, as well as a marked increase in p53 levels, suggestive of enhanced apoptosis (Fig. R.32B). Interestingly, simultaneous expression in the silenced MCF7 cells of the catalytically inactive GRK2-K220R mutant did not rescue the blockade of tumour induction, once more indicating that kinase activity was required for the tumour-promoting effect of GRK2 in vivo (Fig. R32C).

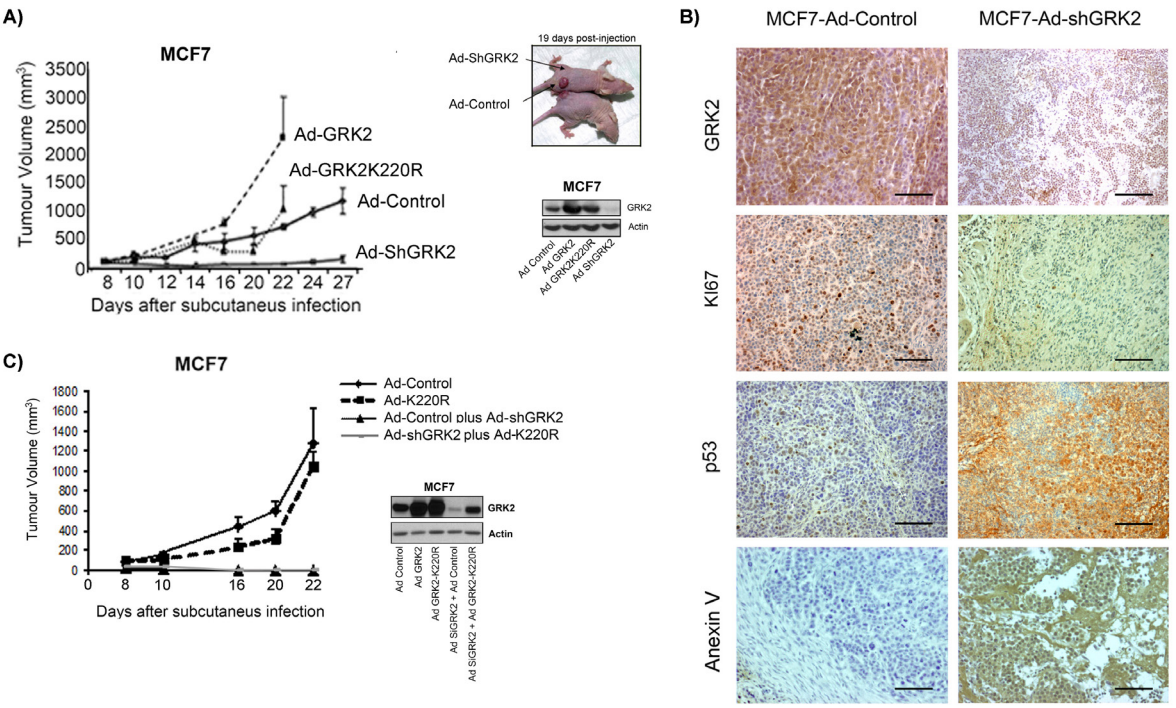


Figure. R. 32. GRK2 depletion abrogates *"in vivo"* tumour progression. MCF7 cells were infected with the indicated adenoviral constructs, and GRK2 levels verified by western blot prior to subcutaneous injection in nude mice. Evolution of tumour volume (mm^3) from 6-10 mice per condition was determined in panel A (*p<0.05, **p<0.01, ***p<0.001). B) Immunohistochemical analysis of the expression of GRK2, KI67, p53 and anexinV in tumour sections was performed 8-days post-injection. (Scale bar: 100 μm). C) The effect of GRK2 downmodulation was not rescued by reintroduction of a kinase-inactive mutant .

12.2. GRK2 modulates tumour growth promoted by transformed breast cancer cells harbouring p53 mutations.

We next asked whether the in vivo ability of GRK2 to promote tumour growth and, conversely, the inhibitory effect on this process of GRK2 silencing was restricted to luminal cells with high levels of GRK2, wild-type p53, ER-positive and mutated PI3KCA such as MCF7 cells, or was also taking place in breast cancer cells with other molecular signatures. To address if such GRK2 effects were independent of the p53 status, we used MDA-MB-468 transformed cells (high GRK2 levels, ER negative, HER2 negative, mutated p53, mutated PTEN), in which we had previously observed that GRK2 up- or down- regulation alters proliferation in culture in opposite ways (Fig. R.23A). Interestingly, whereas tumours induced by MDA-MB-468 cells over-expressing wild-type GRK2 were slightly higher in size compared to those formed by control cells, kinase down-modulation using the adenoviral silencing construct markedly inhibited tumour formation in vivo (Fig. R.33A). Furthermore, GRK2 over-expression in the basal-like MDA-MB-231 cells (low GRK2 levels, ER negative, HER2 negative, mutated p53 and without mutations in the PI3K/AKT pathway) strongly induced higher tumour growth compared with tumours formed by MDA-MB-231 control cells (Fig. R.33B).

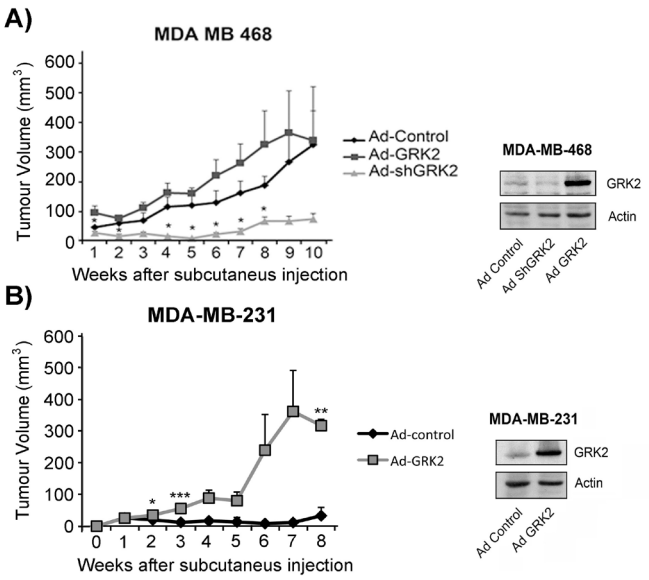


Figure. R. 33. GRK2 influences *"in vivo"* tumoural growth of transformed cells bearing mutant p53. MDA-MB-468 (A) or MDA-MB-231 (B) cells were infected with the indicated adenoviral constructs and implanted in 6 mice/group as in previous Figures. Size of tumour masses raised from subcutaneously implanted cells were measured for 10 weeks after injection. Data are mean ± SEM of 6 mice per group (*p<0.05). Levels of GRK2 were monitored by western blot before injection in mice. (*p<0.05, **p<0.01, ***p<0.001).

Taken together, these results showed that GRK2 is a key modulator of tumour growth promoted by either luminal or basal breast cancer cells, even when the extent of GRK2 expression in the two different lineages is different. Moreover, the ability of GRK2 to promote breast cancer development in vivo is independent of the p53 status, at least in some contexts, pointing at the existence of different GRK2-dependent regulatory mechanisms governing these processes. Overall, these data suggest a more general role for GRK2 in tumoural transformation.

13.

GRK2: a new player in breast cancer invasive migration?

Cancer progression and outcome are conditioned by the acquisition of two key abilities by tumor cells: the growth and survival capability that trigger resistance to therapy and the invasion into host tissues resulting in local or systemic metastatic dissemination. Although both processes are often studied separately, the underlying mechanisms appear to be governed by similar signaling nodes. These overlapping pathways are usually controlled by growth factor and chemokine receptors, p53 mutations and components of the Ras/ MAPKs/ PI3K axis, among others (Alexander & Friedl, 2012). Mdm2 also promotes cell migration and invasion through the ubiquitination and degradation of E-Cadherin (Yang et al., 2006) as well as by positively regulating Slug and MMP9 (Chen et al., 2013b; Jung et al., 2013a) and GRK2 has been shown to promote epithelial cell migration in both kinase-dependent and independent manners, by interacting with and/or phosphorylating relevant key drivers of cell motility such as ezrin, paxilin, tubulin, GIT1 or HDAC6 (reviewed in Penela et al., 2014). Based on these evidences, we set up to investigate a possible role for GRK2 in the invasive migration of breast cancer cells.

13.1. GRK2 expression is enhanced in metastatic Infiltrating Ductal Carcinoma (IDC) samples from patients

Previous data from our lab in collaboration with the Oncology and Pathological Anatomy Departments of the Hospital Universitario La Paz (Dr. David Hardisson) showed that GRK2 protein levels, as assessed by western blot analysis, were increased in an important proportion (41%) of a limited cohort (27) of infiltrating ductal carcinoma patients (Alicia Salcedo, Doctoral Thesis). Interestingly, an inmuno-histochemical analysis of paraffin-embedded sections of an independent cohort of 49 metastatic infiltrating ductal carcinomas showed that GRK2 up-regulation was present in circa 80% of the primary tumours from patients that underwent regional lymph node colonization (Fig. R.34.A), (i.e patient's lymph nodes near the primary tumour tested positive for malignancy). Moreover, all of the AKT-positive metastatic samples displayed high levels of GRK2, which support the previously proposed AKT-dependent control of GRK2 expression in a human pathological context (Fig. R34B).

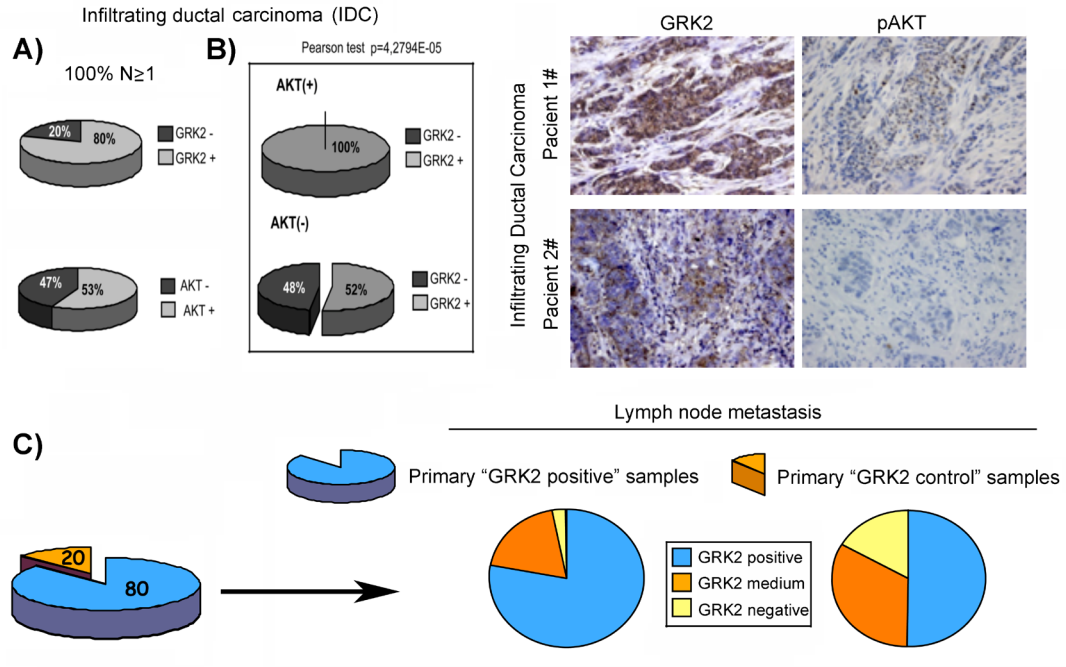


Figure R.34: GRK2 expression is enhanced in metastatic infiltrating ductal carcinoma samples from patients. A) Increased pAKT in metastatic infiltrating ductal carcinomas correlates with increased GRK2 levels. Primary tumour samples of 49 patients were analyzed by immunohistochemistry to detect GRK2 and p-AKT with specific antibodies. Samples were scored as positive (moderate or strong staining, +) or negative (none or weak staining,-) for GRK2 and p-AKT levels. B). Samples were then stratified by pAKT levels and the distribution of GRK2 groups plotted in a pie chart (p = 4,24 E-05; Pearson test). Representative sections of GRK2 and pAKT staining of two patients are shown. C) Final metastasis of the 49 infiltrating ductal carcinomas was analyzed by immunohistochemistry to detect GRK2 levels with specific antibodies. Samples were scored in as positive (strong staining), medium (moderate or weak staining) and negative (none staining) for GRK2 levels.

Localized spread to regional lymph nodes near the primary tumour is indicative of worse prognosis, being the initial step of the metastatic cascade (Cox et al., 2008). Interestingly, metastatic samples from these primary tumours either retained or acquired enhanced GRK2 levels, (Fig. R.34C) strongly arguing for an important role of GRK2 in the invasive process.

13.2. GRK2 over-expression changes the molecular profiling of breast cells towards a mesenchymal-like phenotype.

We sought to determine whether moderate and stable over-expression of GRK2 in non-transformed breast cancer cells (184B5) could confer these cells migratory advantages by altering the molecular repertoire related to cell motility and invasion. As shown in figure R.35, GRK2 up-regulation increased the levels of GIT1, a relevant scaffolding protein involved in cell motility and cellular adhesion previously identified as a kinase-activity- independent partner of GRK2 in epithelial cell migration (Penela et al., 2008). Moreover enhanced GRK2 levels also promoted the down-regulation of E-Cadherin, which is key for the progress of the epithelial-mesenchymal transition (EMT) process and a recently described Mdm2-regulated protein (Hazan et al., 2004; Sarrió et al., 2009; Yang et al., 2006). Moreover, Vimentin, a mesenchymal marker up-regulated in many epithelial cancers (Markiewicz et al., 2012), was also enhanced upon over-expression of GRK2 (Fig. R.35), in line with the notion that altered GRK2 expression levels might alter migratory responses and potentiate the invasion process.

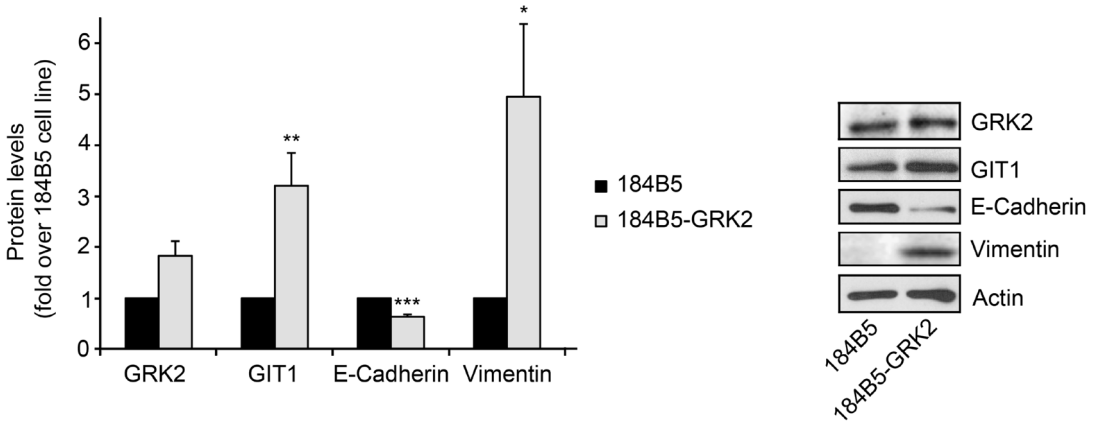


Figure R.35: Moderate and stable overexpression of GRK2 in non-transformed breast cells (184B5) regulates proteins implicated in tumour migration and EMT. A) Parental or GRK2-stable over-expressing 184B5 cells were lysed and steady state levels of GRK2 and several factors that impel tumour migration and/or invasion such as GIT1, E-Cadherin and Vimentin were quantified upon immunodetection with specific antibodies. The amount of each one protein in 184B5 cells was taken as 1. Data are mean ± SEM of 4 independent experiments. B) A representative gel is shown.

13.3. GRK2 favors the chemotactic response of breast cells with different invasive potential

Chemotaxis integrates complex steps coordinated by transiently activated signaling networks: the detection of asymmetric extracellular cues (chemical gradients) by membrane receptors and the spatially controlled reorganization of intracellular signaling effectors and the cytoskeleton machinery, leading to cell polarization, membrane protrusion and the generation of dynamic adhesion and traction forces required to move the cell towards the signal source (Frame et al., 2002). As GRK2 is engaged in many of these processes that enable normal cell migration of epithelial cells (Penela et al., 2014b), we sought to analyze the effects of altering GRK2 expression in migration of breast cells with different invasive potential. Thus, stable over-expression of GRK2 to different extents in the non-transformed breast cancer cell line 184B5 enhanced cell migration in response to EGF or Heregulin (Fig. R.36A). On the other hand, adenoviral infection of GRK2 in the highly metastatic MDA-MB-231 cells also potentiated their migratory capability upon Heregulin stimulation, whereas the knock down of the kinase strongly impeded the chemotactic response (Fig. R.36B). We next asked whether the ability of GRK2 to modulate migration was restricted to EGF and Heregulin treatments (acting through tyrosine kinase receptors) or was also taking place upon other chemotactic stimuli signaling through GPCR receptors. As shown in figure R.36C, GRK2 silencing clearly disrupted chemotactic migration of MDA-MB-231 cells induced in response to CCL21, a chemokine reported to mediates actin polymerization, pseudopodia formation, and invasive responses in this cell type (Müller et al., 2001).

These data strongly suggested that GRK2 is a key player of epithelial cancer cell migration. Interestingly however, random (non-directed) cell migration of MDA-MB-231 cells was inversely affected by the knockdown of GRK2 as assessed by two different experimental approaches (Fig. R.37A and B), suggesting that GRK2 levels would differentially effect directed and random migration processes (see Discussion) (Penela et al., 2014b).

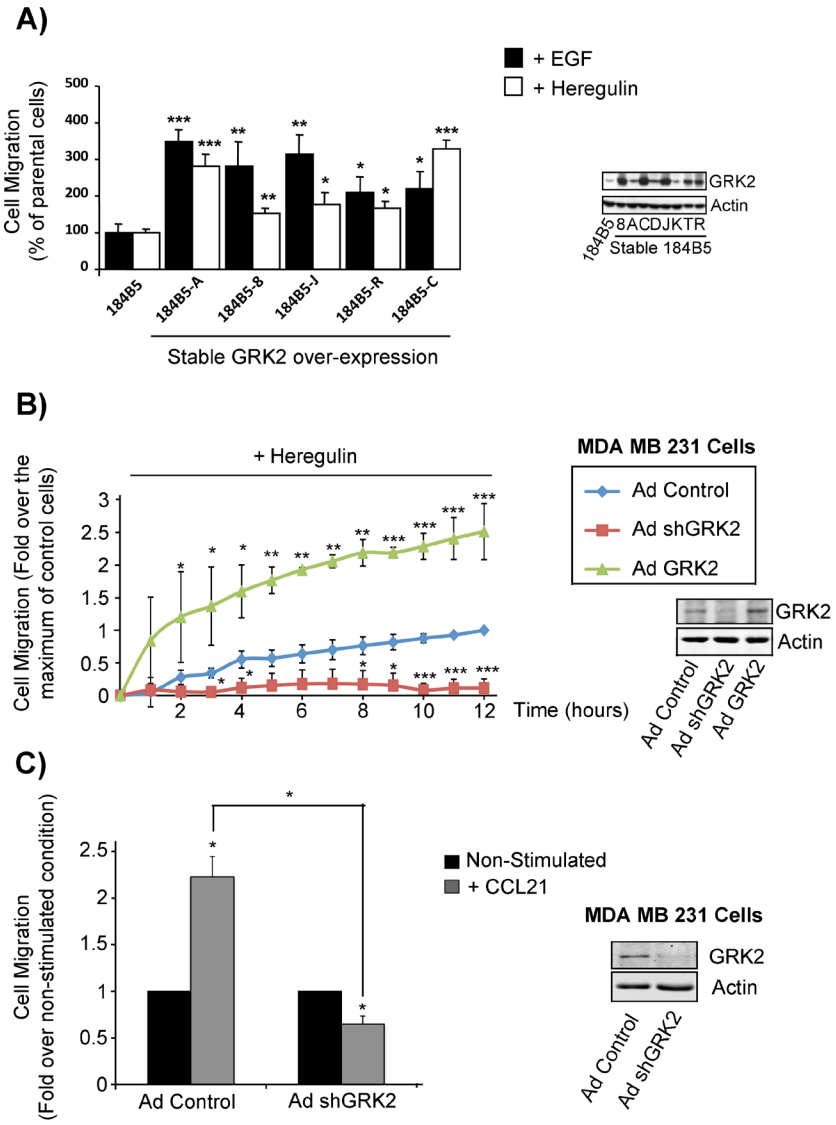


Figure R.36: GRK2 increases migration of different breast cell lines with different metastatic potential. A) 184B5 cells stably over-expressing different amounts of wt GRK2 (showed in the blot) were serum-starved and plated on Transwell filters. Cell migration towards Heregulin and EGF was assessed as detailed in Materials and Methods. Data are the mean ± SEM of 4–6 independent experiments performed in duplicate. (*p<0.05, **p<0.01, ***p<0.001). B) MDA-MB-231 cells were infected with the adenoviral constructs of GRK2, shGRK2 or with a control adenovirus. Cell migration towards Heregulin was assessed using the xCellLigence system. Raw data were normalized to a (0–1) scale through division of all data by the maximum value obtained. Subsequently, random migration (SF) signals were subtracted from the positive (Heregulin) control counterparts per experiment to obtain a pure chemotactic signal. Graphs represent normalized data over migration of control cells. All results originate from three independent duplicate experiments and are represented as the mean +- SEM. (*p<0.05, **p<0.01, ***p<0.001). C) Cellular migration to CCL21 was significantly decreased upon reduction of GRK2 expression in MDA-MB-231 cells. MDA-MB-231 cells infected with an adenoviral-GRK2 shRNA construct or a control adenovirus were serum starved and plated on Transwell filters with a bottom chamber covered with serum-free media with or without CCL21. Cell migration was assessed and data (mean ± SD of 2 independent experiments performed in duplicate) normalized to the non-sitmulus condition. (*p<0.05, **p<0.01, ***p<0.001).

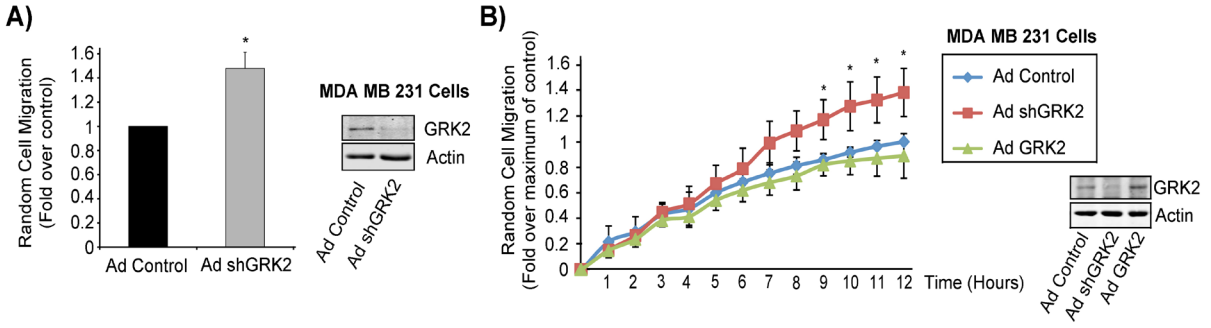


Figure R.37: Random cell migration is negatively controlled by GRK2. A) MDA-MB-231 cells were infected with different adenoviral constructs (GRK2 wt, shGRK2 or a control) and random cell migration was measured by transwells assays (A) or using the X-CellLigence system as described in Methods section. (B). Migrated cells were counted and normalized to the control condition. Data are mean ± SD of two independent experiments performed in duplicates. (*p<0.05, **p<0.01, ***p<0.001).

13.4. GRK2 potentiates cell invasion through the basement membrane

Breaching of the basement membrane, a highly cross-linked meshwork that separates epithelial cells from connective tissues, is the first physiological barrier to cancer-cell migration, because it does not contain pores large enough for passive invasion (Poincloux et al., 2009). Tumour cell invasion across tissue boundaries and metastasis requires the breaking of this membrane and extracellular matrix (ECM) remodeling. These processes depend on the capacity of cancer cells to form invasive structures called invadopodia. However, the present knowledge of how invadopodia form and function is very limited.

In order to start delineating the possible contribution of GRK2 to the cell invasion process, we utilized a transwell assay with the upper chamber covered with matrigel, whose composition mimics the basement membrane (mainly constituted by Type-IV collagen, laminin, entactin and heparan-sulphate proteoglycans). Either parental or 184B5 cells stably-over-expressing GRK2 were seeded on top of the matrigel upper chamber and their invasive ability was measured in response to the chemo-attractants EGF or Heregulin. As shown in Figure R.38A, GRK2 strongly potentiated the basally low directed invasive response to both stimuli. Conversely, knockdown of GRK2 in the highly invasive MDA-MB-231 cell line profoundly decreased matrigel cell invasion (Figure 38.B and C) using fetal serum bovine as the chemo-attractant. Interestingly, the inhibitory effect of GRK2 silencing is even stronger than the invasion blockade promoted by the down-modulation of MT1-MMP, a membrane-anchored metalloproteinase that is crucial for the breakdown of both BM and ECM (Poincloux et al., 2009). These data suggested a dual role of GRK2 in the invasive migration of breast cancer cells, promoting both cell migration and the disruption of the basement membrane.

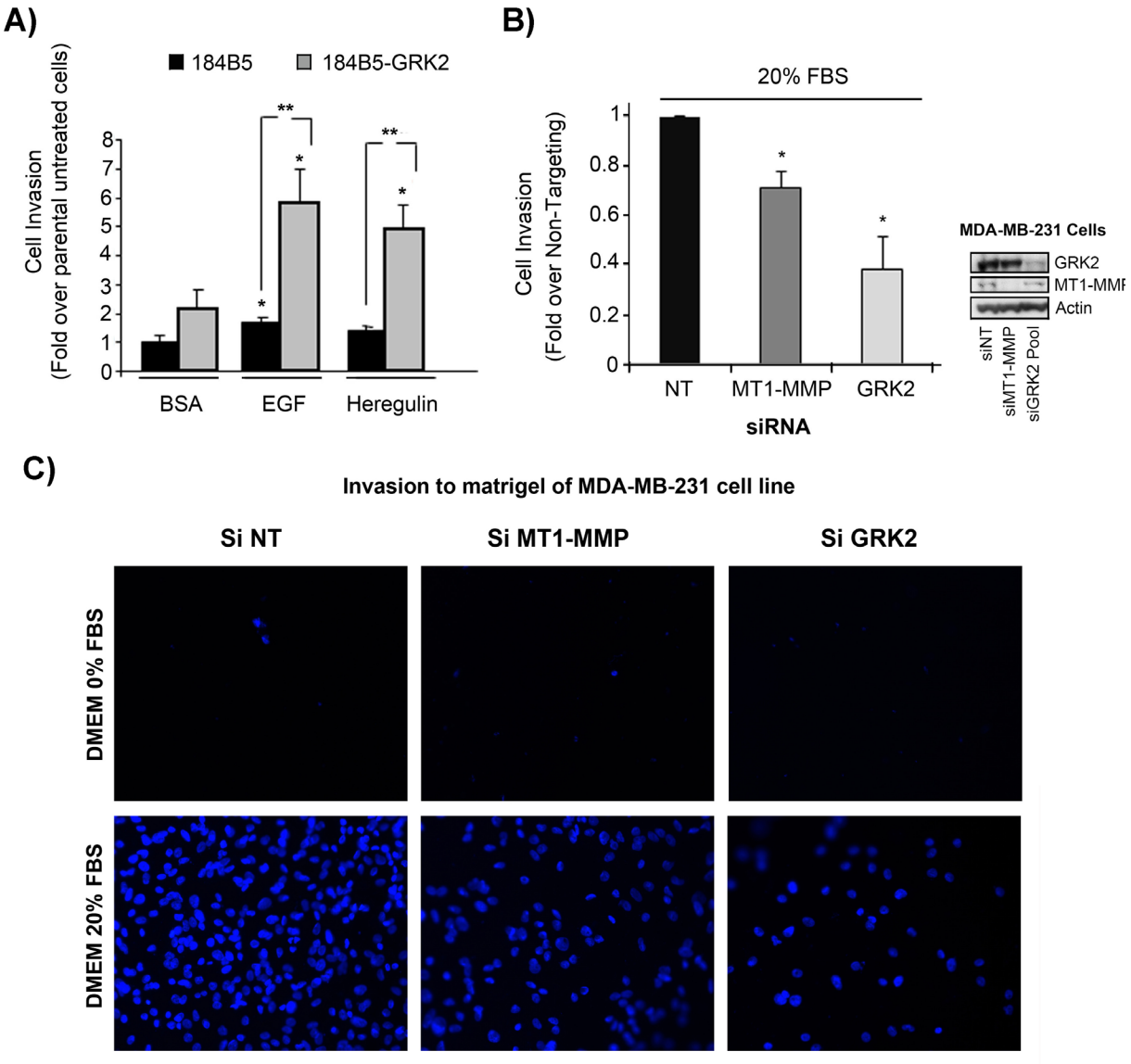


Figure R.38: GRK2 is necessary for the invasion through matrigel in both normal and transformed breast cells. A) Parental or GRK2-over-expressing 184B5 cells were seeded on Transwell filters precoated with 250 μg/mL matrigel and cell invasion was assessed in response to EGF and heregulin as detailed in Materials and Methods. Data are the mean ± SEM of 4–6 independent experiments performed in duplicate. (*$p<0.05$, **$p<0.01$). B) MDA-MB-231 cells were transfected with different siRNAs (non-targeting, MT1-MMP or GRK2) using the Cell Line Nucleofector System (as described in Materials and Methods). After 24 hours, cells were serum-starved for 4 hours and seeded on Transwell filters precoated with 250 μg/mL matrigel and cell migration was assessed in response to 20% FBS. Data are the mean ± SD of 2 independent experiments performed in duplicate. (*$p<0.05$). Transfection efficiency was analyzed by western blot using specific antibodies towards MT1-MMP, GRK2 and Actin as the loading control. C) A representative experiment from B is shown.

13.5. GRK2 is also relevant for extracellular matrix remodeling and oriented cell invasion in 3D models.

After basement membrane crossing, cancer cells infiltrate local tissues by trafficking through a stromal extracellular matrix (ECM). Therefore, we next assessed the contribution of GRK2 to such ECM invasion, using a highly metastatic cell line and conditions that mimic the 3D tumor environment. Thus, a OrisTM Collagen I Cell Invasion Assay was performed (see Materials and Methods section), using MDA-MB-231 cells that stably express the nuclear reporter protein Histone-2B(H2B)-EGFP (provided by Dr.P. Chavrier's lab) and reconstituted acid-extracted type I collagen to mimic the architecture of native type I collagen fibrillar networks (ECM). MDA-MB-231-H2B cells were then treated with siRNAs specific for GRK2, MT1-MMP or with non-targeting siRNA and their capacity to invade the collagen I-filled, cell-free central portion of the well over a 2-day culture period was measured (Fig. R.39A). As previously shown (Rey et al., 2011), knockdown of MT1-MMP led to 40% reduction of the invasive capacity of MDA-MB-231 cells (Fig. R39A and B). Remarkably, silencing of GRK2 by 2 different siRNAs also resulted in a strong inhibition of ECM invasion of these cells. To rule out the possibility that the invasion defects observed in the GRK2-silenced transformed breast cancer cells were due to the GRK2-dependent contribution to cell proliferation, we analyzed the proliferation rate by measuring the total area of the well occupied upon 2 days of experiment. We could not detect any differences in cell growth of the different siRNA-treated cells in this type of experimental approach (Fig. R.39C), suggesting that the observed effect of GRK2 in the 3D collagen type I invasion was independent of its role on breast cancer cell growth. To confirm this result, an independent experiment using a similar strategy was performed based on formation of multicellular spheroids by MDA-MB-231 cells (see Methods). We reconstituted a 3D in vivo-like environment for invasive breast cancer cells by embedding these multicellular spheroids of MDA-MB-231 cells within 3D gels of native acid-extracted type I collagen. As shown in figure R.40A, MDA-MB-231 cells invaded and disseminated in the surrounding matrix with a radial pattern over a 2-day culture period. Interestingly, GRK2 down-modulation significantly disrupted this ability, as potently as knockdown of the MT1-MMP matrix metalloproteinase (Fig. R.40A and B), which is a well-established essential factor for ECM remodeling and breast cancer invasion, in absence of significant effects on cell proliferation in such experimental conditions (Fig. R.40C).

Taken together, our findings suggested that over-expression of GRK2 is able to promote invasive migration of breast cells whereas its presence appears to be critical to maintain the invasiveness of highly metastatic breast cancer cells through both the basal membrane and three-dimensional extracellular matrix.

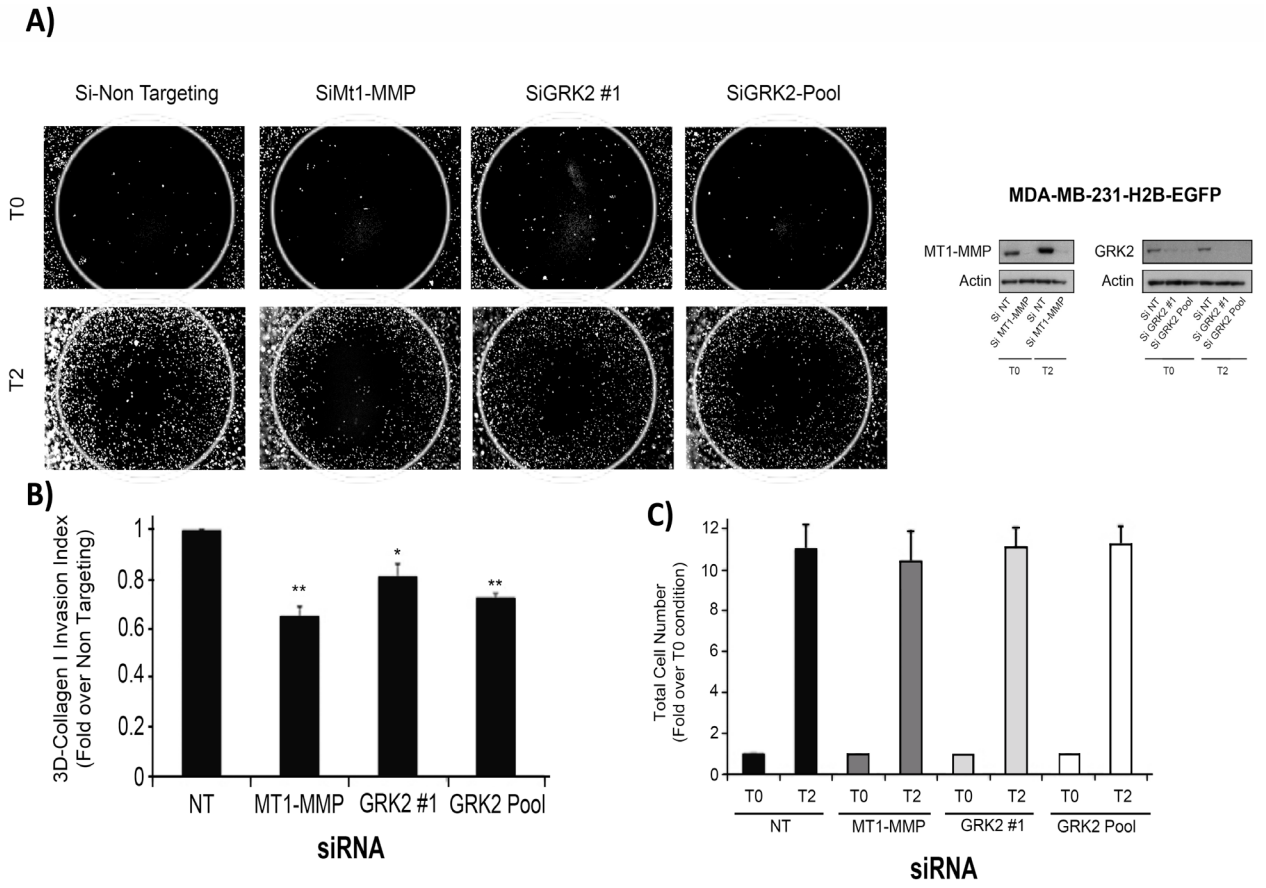


Figure R.39: GRK2 is required for oriented Collagen type I invasion in 3D. A) H2B-EGFP/MDA-MB-231 cells treated with the indicated siRNAs were tested using the Oris™ 3D-Collagen I invasion assay for 60 h. (see Methods section). Immunoblotting analysis of MT1-MMP and GRK2 levels in MDA-MB-231 cells treated with the indicated siRNAs at the time of cell seeding (T0) and 48 h after seeding (T2) is shown. B) Invasion assay index was calculated by thresholding the area occupied by H2B-EGFP nuclei in the detection zone of each well after 48 h of invasion. This index is defined by the total area occupied by H2B-GFP nuclei in unseeded area (detection zone) at the end of the assay (T2) by subtracting the area occupied by H2B-GFP nuclei at the beginning of the experiment (T0). This area of invasion was normalized and results were presented as the fold increase of invasion over the control condition. Values represent mean invasion index ± SEM from 2 independent experiments. (*P<0.05 ;**P < 0.01). C) Cell proliferation is not modified by GRK2 in this experimental aproach. Cell proliferation was measured by calculating the total number of nuclei in the complete well (unseeded + seeded area) at the end of the assay (T2) normalizing by the nuclei number at the beginning of the experiment (T0). Data are mean ± SEM from two independent experiments.

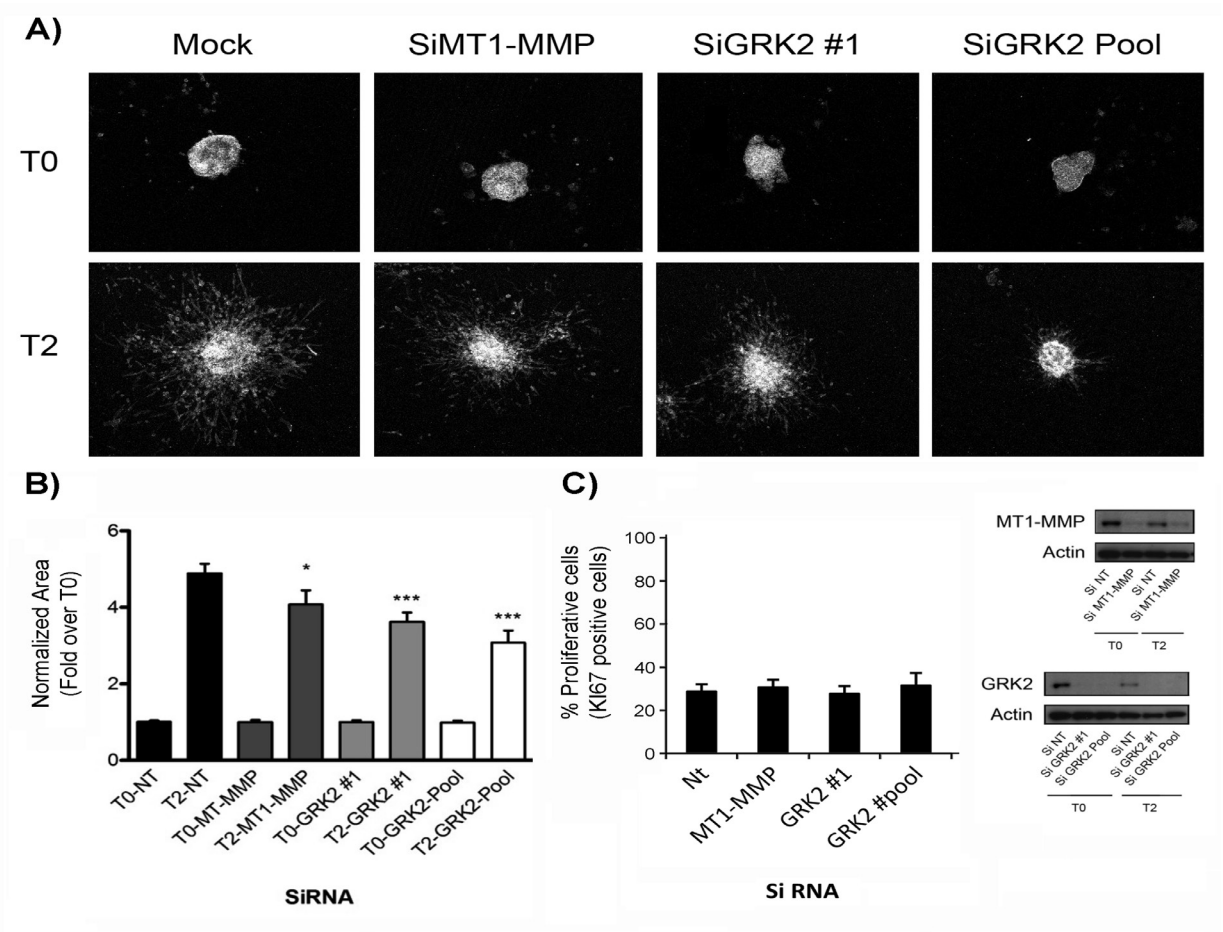


Figure R.40 GRK2 is required for 3D Collagen type I invasion upon spheroids formation. A) Multicellular spheroids of MDA-MB-231 cells (3 × 10^3 cells) were prepared in hanging droplets after nucleofection with MT1-MMP or GRK2 siRNAs and embedded in 3D type I collagen gels (2.2 mg/ml, prepared from acid extracts of rat tail tendon). After 2 days, samples were fixed and stained with fluorescent phalloidin to label F-actin. Invasion was monitored by confocal microscopy at 0-day (T0) and after 2-days of culture (T2). A Western blot of the cell lysates at T0 and T2 was performed to analyze the levels of MT1-MMP and GRK2. B) Invasion area in 3D type I collagen gels was calculated by estimating the diameter of spheroids at T0 and T2. Spheroid diameters were averaged and used to calculate the mean invasion area (πr2). For each cell population, mean invasion area at T2 was normalized to mean invasion area at T0, and plotted with error bars representing ±SEM (N = 2 independent experiments for siRNA knockdown, analyzing a total of 15–35 spheroids for each cell population. (*P < 0.05; ***P < 0.001). C) The number of proliferative cells was calculated after immunoflorescence staining of the proliferative marker KI67. KI67 positive cells (%) in the invasive area was calculated as described in Methods section.

13.6. GRK2 knockdown impairs the activity of ECM-degrading invadopodia.

Migration and invasion are related processes that however relay on separated molecular programs responsible for their different outcome. Migration is defined as the movement of cells from one location to another on a particular substrate such as basal membranes, ECM fibers or plastic plates that proceeds without physical barriers. Therefore, migration is occurring on 2D surfaces or within 3D structures without any obstructive fiber network. Invasion is defined as cell movement through a 3D matrix or across basal membranes, which are accompanied by a restructuring of the environment. Thus, invasion processes requires adhesion, either proteolysis or squeezing strategies and migration through the ECM. We thus explored whether the GRK2-dependent invasive effect could involve the modulation of the specific capability of breast cancer cells to degrade the matrix through the formation of invadopodia.

To discriminate the contribution of GRK2 to cell motility from that related to invasive migration of MDA-MB-231 cells, we set up a time-lapse video-microscopy assay using the stable MDA-MB-231-H2B-EGFP cell line seeded onto either a 2D collagen substrate or a 3D collagen-I matrix. Quantification of random cell motility was analyzed by calculating the displacement index of H2B-EGFP-labeled nuclei recorded over 48-60 h. As should be expected, silencing of the metalloproteinase MT1-MMP disrupted only the random invasive motility in 3D conditions (Fig. R.41A and B), but did not alter the 2-D motility of these cells. Interestingly, down-modulation of GRK2 resulted in a similar pattern of effects (Fig. R.41A and B).

Overall, these data suggest that impaired invasion of GRK2-depleted MDA-MB-231 cells in 3D collagen matrix is likely the consequence of a defect in the ECM-degradation ability of the cells, which finally result in the disruption of breast cancer cells movement. The fact that silencing of GRK2 was not altering random cell motility onto a collagen type I surface is consistent with previous results of our laboratory indicating that migration of different epithelial cells towards collagen fraction IV was unaltered by alterations in GRK2 levels, which suggests that the pro-migratory effect of GRK2 is context specific and does not affect the overall cell motility (Penela et al., 2008).

When tumor cells are grown on a 2D matrix substratum in order to reconstitute tumor cell-Basal Membrane interactions in vitro, matrix proteolysis is restricted to ventral cell surface structures called invadopodia, which correspond to actin and cortactin-rich finger-like membrane protrusions enriched in MT1-MMP (Artym et al., 2006a; Poincloux et al., 2009).

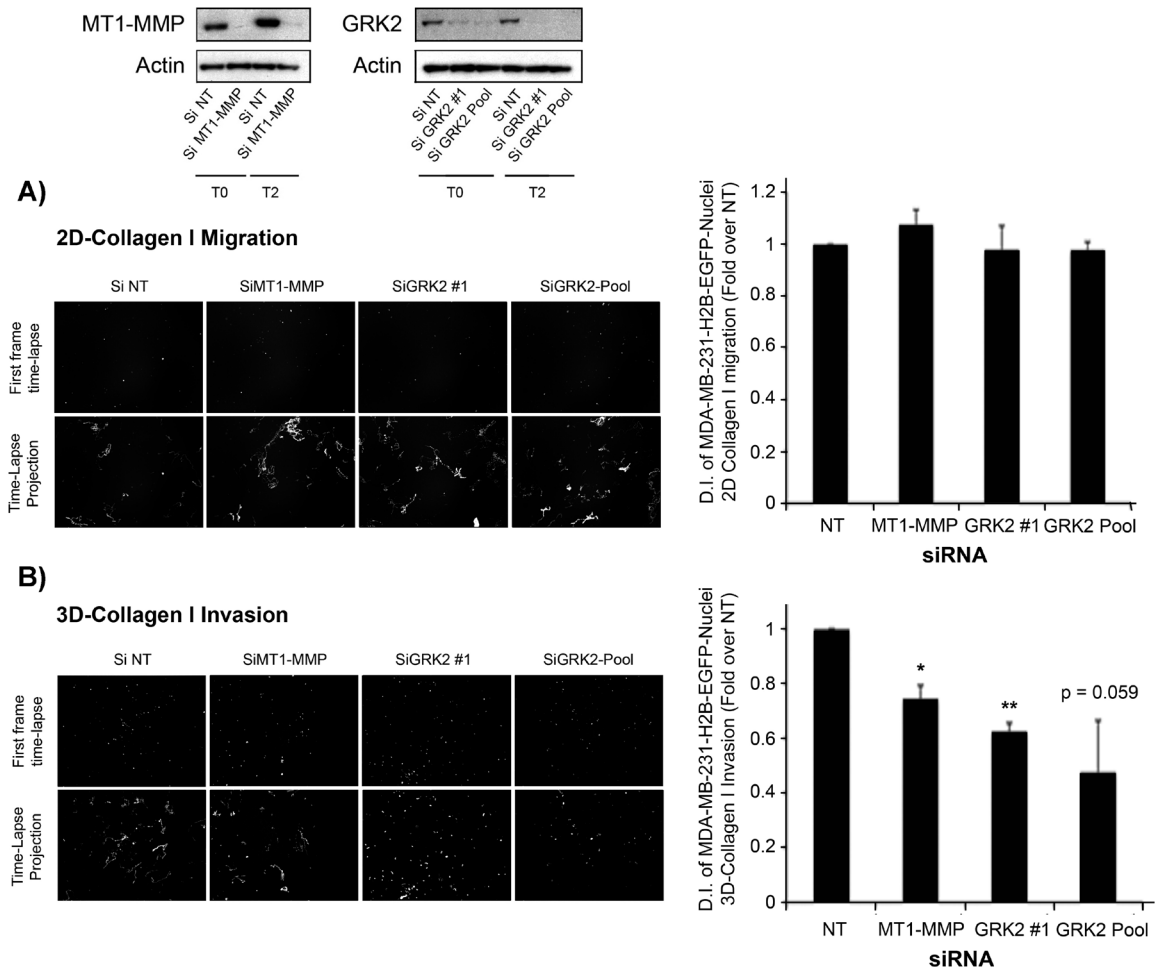


Figure R.41: The absence of GRK2 does not affect migration in 2D Collagen type I matrix but disrupts invasive motility of the cells in 3D conditions. A) H2B-GFP-expressing MDA-MB-231 cells treated with the indicated siRNAs (2000 cells/well), were seeded in 2D- type I collagen-coated wells and time-series of GFP signal were recorded at 15-min intervals for 48 h over 2 random fields. Representative images corresponding to the first frame and maximum projection of the time-series are shown for each cell population. Displacement Index (DI) of H2B-EGFP nuclei are represented as the mean DI ± SEM from two independent experiments (in duplicate) with DI of mock-treated cells set to 1 (*P<0.05 **P < 0.01) B) H2B-EGFP-expressing MDA-MB-231 cells treated with indicated siRNAs (2000 cells/well), were embedded in a 3D-matrix of acid-extracted type I collagen, and time-series of GFP signal were recorded at 15-min intervals for 48 h over 2 random fields. Representative images corresponding to the first frame and maximum projection of the time-series are shown for each cell population and DI of H2B-GFP nuclei are represented the mean DI ± SEM from two independent experiments (in duplicate).

To explore whether GRK2 might alter invadopodia activity, (Rey et al., 2011)we performed a localized degradation assay by culturing MDA-MB-231 cells on FITC-gelatin. As previously shown (Castro-Castro et al., 2012; Rey et al., 2011), knockdown of MT1-MMP markedly reduced the proportion of "degradative" cells (from 25-30% to 5%) (Fig. R.42A-C) and invadopodia-dependent gelatin degradation to 10-20% of the capacity of cells treated with a non-targeting siRNA (Fig. R.42D). Remarkably, knockdown of GRK2 with

different siRNAs (Fig. R42.A), although it did not affect the morphology or spreading of MDA-MB-231cells on gelatin (Fig. R.42B), led to a significant inhibition (50-70%) of both invadopodial matrix degradation and the number of cells displaying degradation abilities. (Fig. R.42C-D). Overall, we can conclude that GRK2 is required for two-dimensional matrix proteolysis in the highly invasive human breast carcinoma cell line MDA-MB-231.

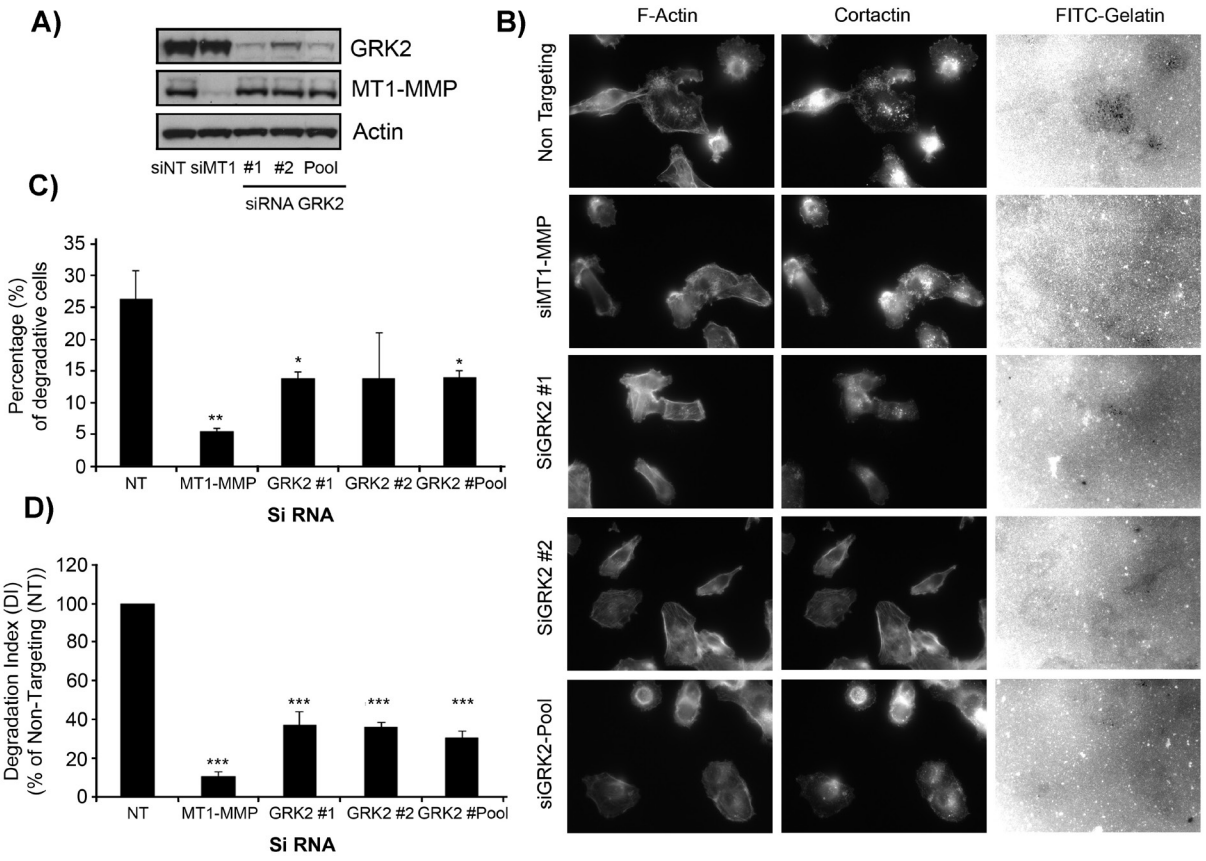


Figure R.42: Depletion of GRK2 strongly decreases the capacity of MDA-MB-231 cells to degrade gelatin. MDA-MB-231 cell were transfected with different siRNA (non-targeting, MT1-MMP and different oligos for GRK2) by using the Cell Line Nucleofector®. Lysates were analysed by western blot to check the depletion of GRK2. A) MDA MB231 cells transfected with the indicated siRNAs were incubated on FITC-gelatin for 4 h, fixed and stained for F-actin and cortactin. Graphs depicting the percentage of degradative cells (B) and the degradation index (C) of MDA-MB-231 cells treated with indicated siRNA. Values represent means ± SEM from three independent experiments. *P<0.05; **P < 0.01; **P<0.001 (compared to siRNA-non targeting transfected cells)

DISCUSSION

Numerous stress signals have been shown to cause p53 accumulation, mainly by means of the inhibition of Mdm2-dependent degradation of the protein. We report herein that GRK2, known to be a relevant modulator of diverse cellular functions by mechanisms involving either phosphorylation of different substrates or its ability to act as scaffold protein (Evron et al., 2012; Penela et al., 2010a; Ribas et al., 2007), can weaken the efficacy of cellular stress responses by reinforcing the activity of Mdm2 and safeguarding it from inhibitory mechanisms. We find that increased GRK2 levels in breast cancer cells have a relevant impact on cell proliferation, survival and anchorage-independent growth and on tumour growth promotion in vivo. Interestingly, such outcomes are clearly dependent on its kinase activity, since expression of GRK2-K220R, a point mutant that lacks catalytic activity, is not able to mimic the effects of the wild-type protein, In this context, we put forward HDAC6, a recently described novel GRK2 substrate (Lafarga et al., 2012a), and Mdm2, new substrate identified thorough this thesis, as relevant direct targets underlying the effects of GRK2 on luminal breast cancer cells through the control of p53.

1.

Concurrent up-regulation of GRK2, Mdm2 and HDAC6 emerges as a functional module characteristic of luminal breast cancer cells.

Besides oncogenic drivers, alteration of relevant signalling nodes can critically modulate cancer progression-related cellular networks to strength key tumoural hallmarks (Hanahan & Weinberg, 2011). In this sense, GRK2 is emerging as a central signalling hub, not only through its canonical role in the desensitization of many G protein-coupled receptors (GPCR), but also by phosphorylating and/or dynamically interacting with important modulators/ effectors that are potentially relevant in cell transformation, migration or survival (Penela et al., 2010a).

The present work uncovers that GRK2 protein levels are increased in the abundant group of infiltrating ductal breast carcinomas of luminal type, mainly in those ER-receptor positive (which represent two-thirds of all breast cancers) and in 80% of metastaticIDC. Interestingly, the stimulation of different transduction pathways (oestrogens or EGFR receptors, HER2/ras and PI3K/Akt cascades) known to be hyper-activated in luminal breast cancer cell lines as a result of mutation and/or amplification/over-expression of key

signalling proteins (Eroles et al., 2012), not only correlates with increased GRK2 levels but appears to converge in promoting enhanced GRK2 expression in transformed and non-transformed breast epithelial cells (Fig.D1). Thus, the presence of estrogens up-regulates GRK2 levels in ER+ MCF7 and T47 cells, whilst the opposite occurs upon oestrogen withdrawal from the culture medium. On the other hand, blocking EGFR signalling in MDA-MB-468 cells down-modulates GRK2 levels, whereas activation of the Her2/Ras cascade triggers enhanced kinase expression in non-transformed MCF10A (basal-like) or 184B5 (luminal-like) cells and in spontaneous tumours formed in MMTV-Her2 transgenic mice, suggesting that up-regulated GRK2 could also be a feature of certain non-luminal types of breast cancer such as the HER2E subtype (The Cancer Genome Atlas Network, 2012).

Our results are consistent with a relevant role for the activation of the Akt pathway in promoting GRK2 up-regulation. The altered molecular signature of the cancer cells where GRK2 is enhanced share genetic alterations in this pathway (PI3KCA, PTEN) or hyper-stimulation of receptors (EGFR, Her2, ER) able to trigger Akt stimulation (Renoir, 2013; Roskoski, 2014). Notably, the MDA-MB-468 cell line, frequently classified as basal A type (Gordon et al., 2003; Neve et al., 2006), displays mutations in this pathway, contrary to other basal breast cancer cell lines that show normal GRK2 expression. Moreover, enhanced Akt cascade activation positively correlates with GRK2 up-regulation in MMTV-Her2 mammary gland tumours, in the mammary gland of Myr-Akt transgenic mice and in 100% of metastatic infiltrating ductal carcinoma samples from patients. We propose that mitogenic activation of AKT triggers phosphorylation of the E3 ligase MDM2 at serine 166 and serine 186 and its subsequent recruitment to the nucleus, thus hampering Mdm2-dependent proteolysis of cytoplasmatic substrates such as GRK2, increasing GRK2 protein levels as previously suggested for some breast cancer and melanoma cell types by (Salcedo et al., 2006). In fact, we observed a clear correlation between modification of GRK2 levels and phosphorylation of Mdm2 at these residues in normal breast cells transfected with the oncogenes Ras/Her2 and in transformed breast cancer cells upon EGFR inhibition. Such post-transcriptional mechanism for GRK2 up-regulation would be consistent with the fact that increased mRNA expression of the ADRBK1 gene has not been reported to our knowledge as a common feature in the context of breast cancer. It should be noted however that canonical transcriptional modulators as estrogens promoted GRK2 up-regulation in ER+ MCF7 and T47D cells with a time course and pattern similar to that of a well-known ER transcriptional target such as Mdm2 or HDAC6 (Azuma et al., 2009; Brekman et al., 2011; Okoro et al., 2013). Therefore, the occurrence of mechanisms acting at this level cannot be ruled out, although ER-alpha activation in breast cancer cells also

triggers stimulation of the PI3K/Akt cascade, by either direct stimulation of the GPCR-30 receptor or plasma membrane nucleation of Er-α/ kinase complexes (Lee et al., 2005).

Interestingly, Mdm2 is also regulated by the Ras-driven Raf/MEK/MAP kinase pathway (Ries et al., 2000). Therefore, both Mdm2 and GRK2 could be found over-expressed in similar tumoural contexts, since Mdm2 has been also found clearly up-regulated in transformed breast cancer cells of luminal ER positive phenotype and in 40%–80% of late-stage metastatic breast tumours, being associated with worse prognosis, increased metastasis and cancer progression (Araki et al., 2010; Lacroix et al., 2006).

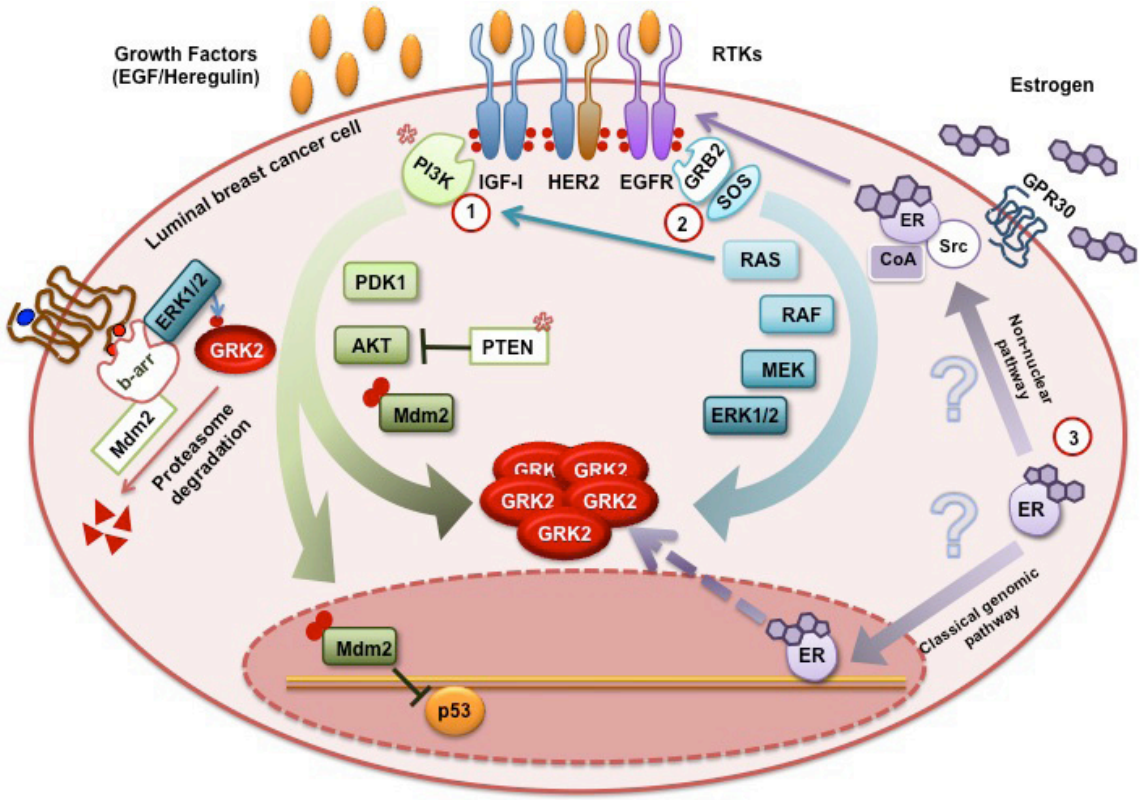


Figure D.1. Oncogenic pathways up-regulate GRK2 in luminal breast cancer cells. Growth factor receptors and/or downstream signalling effectors PI3K (1) and Ras (2), usually mutated or overactivated in luminal breast cancer as well as oestrogen receptors (3) enhance GRK2 expression. * means most commonly mutated proteins in this type of tumours.

Besides Mdm2, we also have found a clear correlation between up-regulated GRK2 levels and over-expression of HDAC6 in luminal breast cancer cells (Fig.D2). HDAC6 has been associated with malignant transformation and invasive motility in breast cancer (Duong et al., 2008; Lee et al., 2008). Interestingly, we have recently reported that GRK2 directly associates with and phosphorylates HDAC6 to specifically stimulate its alpha-tubulin deacetylase activity at discrete cellular localizations in epithelial cells (Lafarga et

al., 2012a). Remarkably, phosphorylation of GRK2 itself at S670 by ERK1/2 in response to stimuli such as EGF was required for the ability of the kinase to phosphorylate and regulate HDAC6 (Lafarga et al., 2012a). Enhanced levels of S670-phosphorylated GRK2 were detected in all cells in our panel that displayed GRK2 up-regulation, what would make more likely a functional interaction between both proteins. As observed for GRK2, HDAC6 levels can be induced by Ras oncogenic signalling in several cell types (Lee et al., 2008) and are transcriptionally upregulated by estrogens in MCF7 cells (Saji et al., 2005b).

Taken together, our data suggest that enhanced activity of different tumour-promoting cascades (PI3K/Akt, estrogens, EGFR, Ras/Neu) in specific breast cancer cells would potentiate the GRK2-HDAC6 signalling module and/or the co-existence of over-expressed Mdm2 and GRK2 proteins, which could be beneficial for tumour development (Fig.D.2). Interestingly, over-activation of growth factor receptor pathways also converges in enhanced GRK2 protein expression in other tumoural cell types such as melanoma cells, where Mdm2 is also up-regulated (Polsky et al., 2001; Salcedo et al., 2006), suggesting that the presence of these new interactomes could be extrapolated to other pathological situations. In this regard, GRK2 has been found up-regulated in thyroid carcinoma, and granulose cell tumours (King et al., 2003; Métayé et al., 2008), where Mdm2 or HDAC6 protein or activities are also enhanced and play a role in the onset of these pathologies (Ding et al., 2013; Haakenson and Zhang, 2013; Kanno et al., 2012; Rayburn et al., 2005; Giaginis et al., 2014). Our data and results from other groups suggest that GRK2 might contribute to the growth of these tumours by positively co-operating with the mitogenic effects of the smoothened receptor, IGF-1, EGF and HER2 receptors or by hampering the TGFβ-triggered anti-proliferative signalling reported in many other cell types (Bliziotes et al., 2000; Dorsam & Gutkind, 2007; Freedman et al., 2002; Gao et al., 2005; Meloni et al., 2006; Penela et al., 2010a; Wan et al., 2003). It is worth noting, however, that the impact of GRK2 expression on specific tumours might differ. Thus, up-regulated GRK2 reduced serum-(or PDGF)-induced cell proliferation in thyroid tumours instead of stimulating it as occurs in breast tumours (Métayé et al., 2008), while a potential role in motility was suggested in line with the pro-migratory effects on mammary tumour cells reported herein. In contrast, down-modulation of GRK2 has been reported in prostate cancers with higher grades of malignancy (Prowatke et al., 2007) and hepatocarcinoma cell lines, in which increased GRK2 levels attenuate IGF-1-dependent signalling and cell growth (Fu et al., 2013; Wei et al., 2013; Zheng et al., 2012). Overall, the physiological outcome of altered GRK2 expression would depend on the cell-type-specific multi-molecular signalling complexes assembled by growth factors and on the pro-transducer vs pro-desensitizing effect of GRK2 on such complexes (Penela et al., 2010a).

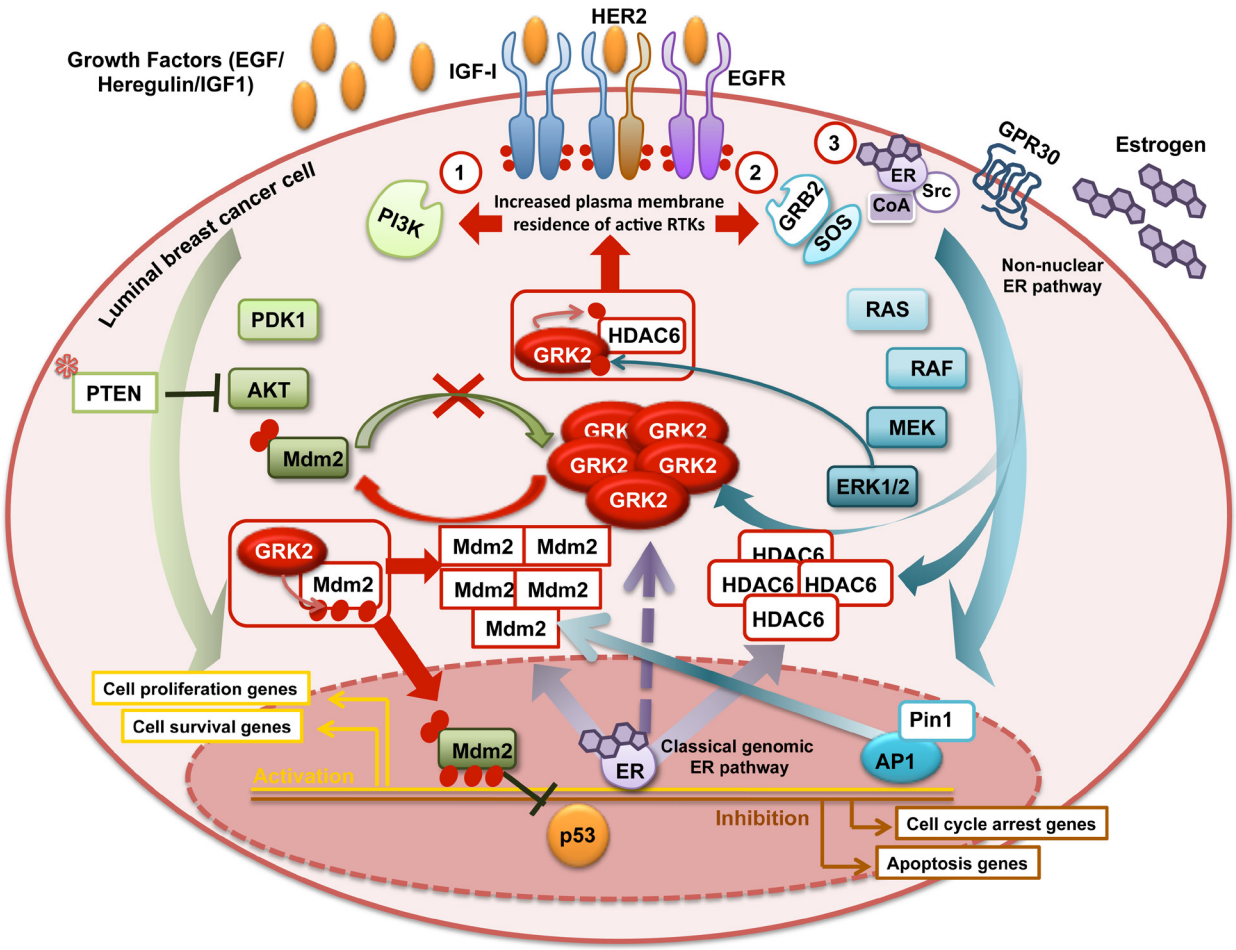


Figure D.2. Luminal breast tumours could benefit from the up-regulation of GRK2,Mdm2 and HDAC6 proteins. Oncogenic pathways responsibles for the up-regulation of GRK2 in luminal breast cancer also promote transcriptional expression and activation of Mdm2 or HDAC6.

2.

GRK2 is a novel kinase activity-dependent activator of Mmd2 that negatively regulates p53

We find that GRK2 levels in transformed luminal mammary cells and in breast cancer cell-derived xenograft tumours inversely correlates with p53 content and activity. Notably, decreased p53 expression is only triggered by a catalytically active form of GRK2 in HEK293 cells and in MCF7-derived xenograft tumours. Moreover in non-tumoural 184B5 cells chronically challenged with EGF, enhanced GRK2 levels reduced the transcriptional activity of p53 and other p53-controlled transcription factors, These data are consistent with previous results from our group showing also a negative correlation between GRK2 levels and p53 expression and induction of apoptosis in the context of cell cycle (Penela et al., 2010b), strongly arguing for a relevant role of GRK2 in the modulation of p53 response in different cellular settings.

p53 protein expression is mainly regulated by the E3 ligase Mdm2, which mediates not only the ubiquitination and degradation of p53, but also controls its transcriptional activity and cell location. Interestingly, whereas Mdm2 regulates proteasome-dependent GRK2 turnover in normal cells, it appears that in a tumoural context over-activation of

growth factor receptor pathways triggers AKT-mediated Mdm2 phosphorylation and its recruitment to the nucleus, hampering this regulatory axis. We propose that in this scenario, GRK2 would interact with and phosphorylate MDM2, establishing a new regulatory feedback loop by which GRK2 potentiates Mdm2 activity towards p53 (Fig.D. 3). Interestingly, enhanced GRK2 protects MCF7 cells from the p53 induction promoted upon treatment with Nutlin3a, a specific inhibitor of the Mdm2-p53 interface, whereas it does not modify p53 levels upon treatment with the E3 ubiquitinase Mdm2 inhibitor HLI373, confirming that the negative effect of GRK2 on p53 depends on Mdm2 ubiquitin ligase activity. Notably, GRK2 not only potentiates MDM2-p53 interaction, but also increases the basal-steady state of Mdm2 and Mdm2 stabilization in HEK 293 cells both in a kinase catalytic-dependent and independent ways. We suggest that the catalytic effects of GRK2 would be triggered by the direct multi-site phosphorylation of Mdm2 at residues S115/116, S260/262 and S351, which are situated within or close to relevant Mdm2 domains such as the p53 binding site (aa1-108), the nuclear location or export sequences (NLS, NES, aa 179-185,190-202 respectively), the central acidic domain (CAD) (aa 243-300) or in between Zn- and RING-fingers (aa 301-335, aa 432-491). These regions are usually selected for post-transductional modifications that affect the functional integrity of the ligase, thereby strongly arguing for a direct role of GRK2 in the regulation of Mdm2 functionality.

It has been proposed that Mdm2 differentially catalyze mono-ubiquitination and poly-ubiquitination of p53 in a dosage-dependent manner (Li et al., 2003), with higher doses of MDM2 favouring poly-ubiquitination and nuclear degradation of p53 (Christopher L. Brooks and Wei Gu, 2006; Li et al., 2003). Conversely, lower doses would trigger p53 multi-mono-ubiquitination that blocks the binding of p53 to DNA and promotes its nuclear exportation to the cytosol, where p53 can stimulate some apoptotic pathways (Marchenko & Moll, 2007; Marchenko et al., 2007). Therefore, by means of Mdm2 stabilization and accumulation, GRK2 could favour p53 poly-ubiquitination versus mono-ubiquitination, resulting in the observed increase of p53 turnover in GRK2 over-expressing breast cells. Interestingly, phosphorylation of Mdm2 residues S115/116 could also affect poly-ubiquitination of p53. These residues are in proximity to domains critical for nuclear shuttling of Mdm2, which inactivation abrogates Mdm2-dependent p53 degradation (Roth et al., 1998; Tao & Levine, 1999). Thus, it is tempting to hypothesize that their phosphorylation by GRK2 could result in alterations of Mdm2 sub-cellular distribution that would impact on the p53 turnover. This is in line with our observation that GRK2 promotes increased nuclear translocation of Mdm2 and destabilization of p53 in a kinase-dependent manner. In addition, S115/116 residues are near the p53 binding site of Mdm2,

suggesting that their modification could increase/stabilize the interaction of both proteins. Further studies with the Mdm2 defective mutants S115/116A will be necessary to unveil the role of GRK2 in this process.

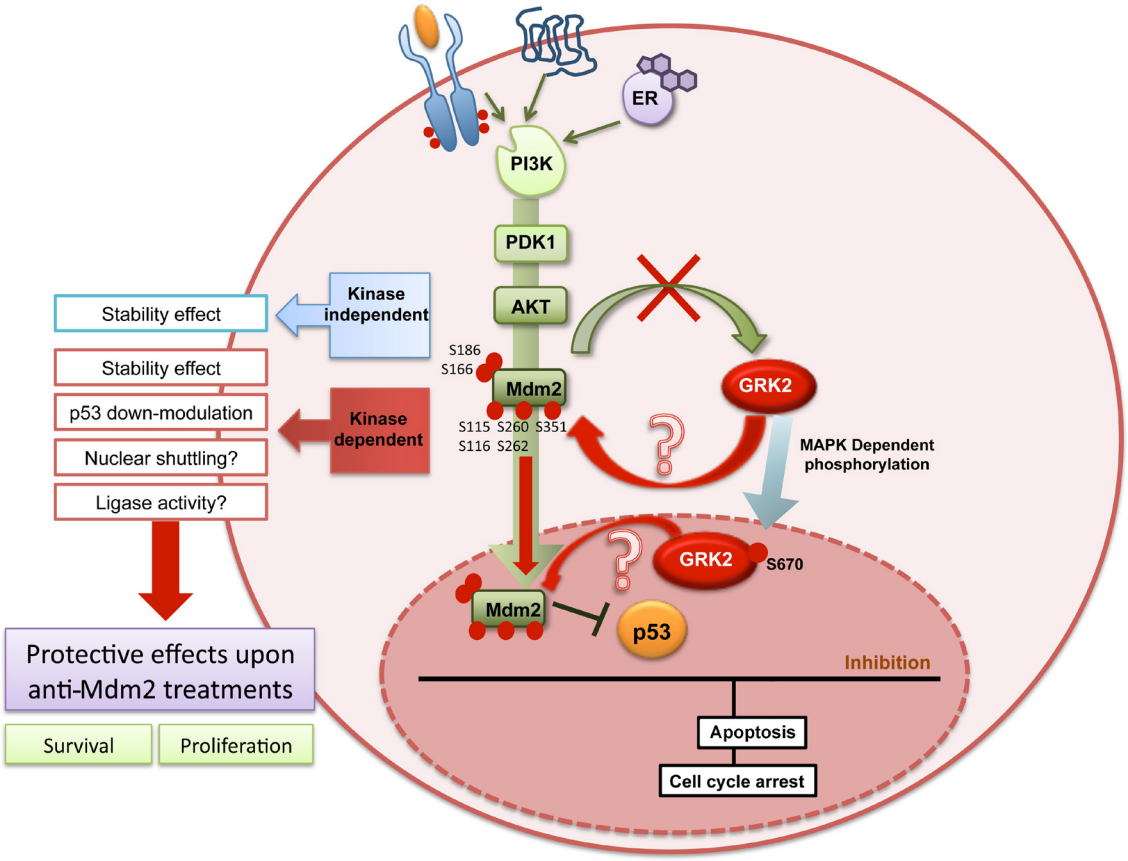


Figure D.3. GRK2/Mdm2 feed-back loop. In some tumoural contexts, GFR, GPCRs and ER would activate the PI3K/AKT pathway, which would result in the AKT-mediated phosphorylation of Mdm2 and the blockade of the Mdm2-dependent cytosolic degradation of GRK2. In such situations, GRK2 would in turn phosphorylate Mdm2 at S115/116 and S260/262 reinforcing Mdm2 stability, the nuclear shutling of the ligase and its E3 ligase activity towards p53.

On the other hand, the central acidic domain (CAD) of Mdm2 is also critical for p53 binding and degradation [Martin et al, 2006, Cheng 2014]. This region undergoes constitutive phosphorylation on multiple serine residues (S229, S232, S240, S242, S246, S253, S256, S260, S262 and S269) in cultured cells (Kulikov et al., 2006). Glycogen synthase kinase 3 (GSK3), casein kinase II (CK2) and casein kinase I (CK1) have been shown to modify some of these sites (Fig I.5). In this context we find that GRK2 is an additional kinase that targets serine 260 or 262 (or both) within the CAD domain. While S260 seems to be targeted preferentially by CK2 (Hjerrild et al., 2001), no kinase had been clearly identified for S262. Interestingly, phosphorylation of S260/S262 by GRK2 might

improve targeting of p53 to proteasome, as this modification lies close to the 257EDV259 motif that is engaged in an intra-molecular interaction with the RING domain, which conceals the C-terminal interface of Mdm2 required for binding to 19S regulatory subunits (Kulikov et al., 2010) However, the multi-serine cluster mentioned above becomes hypo-phosphorylated in response to DNA damage mainly due to the DNA damage-induced downmodulation of GSK3 (Kulikov R et al., 2005). Interestingly, phosphorylation of S242 and S256 by GSK3 is dependent on prior phosphorylation of S246 and S260. It is tempting to speculate that, GRK2 could cooperate with GSK3b in maintaining p53 levels low, priming the Mdm2 modification by this latter kinase in some cellular contexts such as in response to endoplasmic reticulum stress (that promotes p53 degradation) or under nutrient and energy load inducing protein synthesis and growth. On the other hand, GRK2 may functionally replace GSK3b in response to genotoxic stress in order to keep at bay p53 levels and restrict the apoptotic fate of cells in a Mmd2-dependent manner. Thus, while GSK3 is inhibited by an AKT2 and DNA-PK dependent mechanism as part of the genotoxic-driven p53 induction (Boehme et al., 2008), GRK2 is up-regulated under doxorubicin treatment, being feasible it might counterbalance CAD hypo-phosphorylation and p53 stabilization. Moreover, DNA damage and also cell cycle-derived signals contribute to stabilize p53 by promoting multi-site phosphorylation of Mdm2 by CK1, mainly at the CAD by targeting S240, S242, S246, S256 and S260 residues and also the N- and C-terminal of Mdm2 within suboptimal degron sequences that mediate the ligase recognition and destruction by the SCF-bTRCP pathway (Inuzuka et al., 2010). CK1δ phosphorylation of the MDM2 acidic domain also stimulates binding to the p53 core. It is possible that Mdm2 modification by GRK2 might interfere with phosphorylation of CK1, or vice versa, in the CAD and other regions of Mdm2. According to this, we observed that a phospho-defective mutant of Mdm2 at S172, which is a confidently assigned CK1 site in vitro, displayed enhanced phosphorylation by GRK2. Therefore, we hypothesize that under over-activation of oncogenic pathways GRK2-mediated modification of Mdm2 might alter the interaction of the ligase with phosphatases, kinases or partners that target the CAD domain or inhibit the p53/Mdm2 interaction. This notion might be extensive to other regions of Mdm2 modified by GRK2. In particular, the T351 residue phosphorylated by GRK2 lies close to the main sites modified by ATM (S395) or cABL (Y394) after DNA damage. Another potential interplay of interest in the context of cell cycle and checkpoint activation could involve the mutually excluding modification of Mdm2 by CDK2 and GRK2, as suggested by the enhanced GRK2-mediated phosphorylation of a CDK2-phosphodefective mutant of Mdm2 (S213/T216/T218). MDM2 becomes phosphorylated by cyclinA-CDK2 during S-phase progression, and its activity towards p53 is reduced as result of the lower ligase

binding to p53 but higher to ARF protein. It is possible that this regulatory event favors that critical phases of the cell cycle can be on-check by means of the maintenance of a ready-to use pool of p53. In parallel, GRK2 is also phosphorylated by cyclinA-CDK2 during G2 progression, which leads to GRK2 degradation in a Mdm2-dependent manner (previous data of our group). Therefore, it is feasible that CDK2 can switch the repertoire of Mdm2 substrates, by facilitating a complex with GRK2 that could be competent to promote its ubiquitinatation. On the contrary, upon DNA damage, CDK2 activity is inhibited and GRK2 could be engaged in complexes with Mdm2 that would be non-productive in terms of GRK2 degradation but still productive in ligase phosphorylation by GRK2, thereby restricting the extent of Mdm2 inhibition and the p53 responsiveness. In this scenario, up-regulation of GRK2 can interfere in the genotoxic-induced activation of cell cycle checkpoints, contributing to increase genomic instability and cellular transformation.

Overall, high levels of GRK2 could endow the Mdm2 ligase with an intrinsic refractoriness to both DNA damage- and non-genotoxic-triggered inhibition (as inferred from the less sensitivity to Nutlin3a), due to the direct interaction with and phosphorylation by GRK2, and/or the influence of GRK2 on other phosphorylation events that tilt the functional balance of Mdm2 towards a p53 degradation. The impact of GRK2-dependent phosphorylation of Mdm2 on luminal breast cancer might be multifaceted as besides the potential direct effects on allosteric Mdm2 activation and protein sub-cellular localization, it can regulate the binding of other proteins to the CAD domain (regulatory proteins that control Mdm2 targeting to p53) or other regions. As consequence, this model of regulation might adjust the threshold for p53 activation depending on the proliferative or metabolic status of the cell. In addition it might weaken the accuracy of cell-cycle checkpoints and might foster the development of chemoresistance by attenuating the p53 response. How phosphorylation by GRK2 and other kinases is integrated at the same or close sites of Mdm2 in different cellular contexts (basal growing conditions, DNA damage, non-genotoxic stresses as nucleolar, ER, metabolic etc) to reset the outcome of the p53/Mdm2 feedback loop, is a relevant question that remains to be elucidated.

3.

GRK2 mediates breast tumour progression in both wild type and mutant p53 contexts

We find that GRK2 down-modulation similarly inhibited tumour growth of either MCF7 (p53 wild-type) or MDA-MB-468 (mutant p53)-derived xenografts whereas the up-regulation of the kinase increases anchorage-independent growth and tumour growth in vivo induced by MCF7 or MDA-MB-231 (mutant p53) cells. These data may suggest that the ability of GRK2 to modulate breast tumour development in vivo is independent of the p53 status or, alternatively, that it has a double-edged sword role, attenuating p53's tumour-suppressor functions in cells retaining the wild-type factor, but otherwise promoting p53's oncogenic roles in contexts of mutant p53 expression (Fig.D.4). This notion is supported by the fact that Mdm2 and HDAC6 control both wild-type and mutant p53 responsiveness

In wild-type p53 contexts (which account for circa 80% of breast cancers) both the GRK2/Mdm2 axis and the interplay between GRK2/HDAC6 could cooperate to attenuate p53 responsiveness by means of the increase in p53 ubiquitination and degradation and the

decrease in its acetylation and functionality (Fig. D.4). In this regard, it has recently been noted that despite being considered a cytosolic protein, HDAC6 can be found in the nucleus and associated to actively transcribed chromatin (Liu et al., 2012). Interestingly, HDAC6 could interact with wild-type p53 promoting its degradation in a Mdm2-dependent manner, due to removal of acetylation in particular sites that counterbalance protein ubiquitination (Ding et al., 2013). Transcriptional activity of p53 is also reduced by HDAC6 as its binding to p53 displaces the acetylase p300 (Ding et al., 2013), thereby reducing acetylation at the core protein and C-terminal sites. Such modifications are pivotal for proper sequence-specific DNA-binding and important in protein stabilization (Brooks & Gu, 2011). On top of that, the ability of GRK2 to neutralize p53 activity might involve also other functional interactors. We show that enhanced GRK2 expression increases AKT activation upon growth factor stimulation, increases nuclear location of Mdm2 and decreases p53 levels. This could be consistent with an indirect effect of GRK2 on p53 response by potentiating the PI3K/Mdm2/p53 regulatory axis.

In marked contrast, mutant p53 is aberrantly stabilized in tumoural contexts by its uncoupling from Mdm2. In these situations, and opposite to wild-type p53, GRK2 could contribute to protect mutant p53. It has been recently reported that HDAC6 activity promotes the stabilization of mutant p53 in MDA-MB-231 cells through the de-acetylation of Hsp90, which in turn inhibits p53 degradation by blocking Mdm2 (Kovacs et al., 2005; Li et al., 2011b, 2011a). While Hsp90 associates with wild-type p53 only in rare occasions and transiently, many mutant p53 proteins display an abnormal folding in their core domain and form stable complexes with Hsp90, which chaperone activity stabilizes protein folding and prevents protein aggregation. It has been proposed that Mdm2 is non-functionally trapped in ternary complexes with mutant p53 (Hsp90-Mdm2-p53) as presence of Hsp90 might simulate the inhibitory binding of ARF. Given that GRK2 may directly interact with Hsp90 (Chen et al., 2013; Luo & Benovic, 2003) and also phosphorylate and activate HDAC6 (Lafarga et al., 2012), it is tempting to suggest that GRK2 could stabilize mutant p53 by rendering active Hsp90, which could exert a dominant effect on Mdm2 irrespective of its phosphorylation by GRK2. Furthermore, we can not rule out that GRK2 would be unable to phosphorylate Mdm2 in the p53 ternary complex as the region that Hsp90 conceals (S258-S260) spans CAD phospho-sites of GRK2. In coherence with these notions our results show that GRK2 over-expressing MDA-MB-231 cells (mutant p53) are less sensitive to death induced by SAHA, a pan-HDAC inhibitor that preferentially triggers destabilization of mutant p53 by disruption of the HDAC6/Hsp90 regulatory axis (Li et al., 2011a).(Fig.D.4)

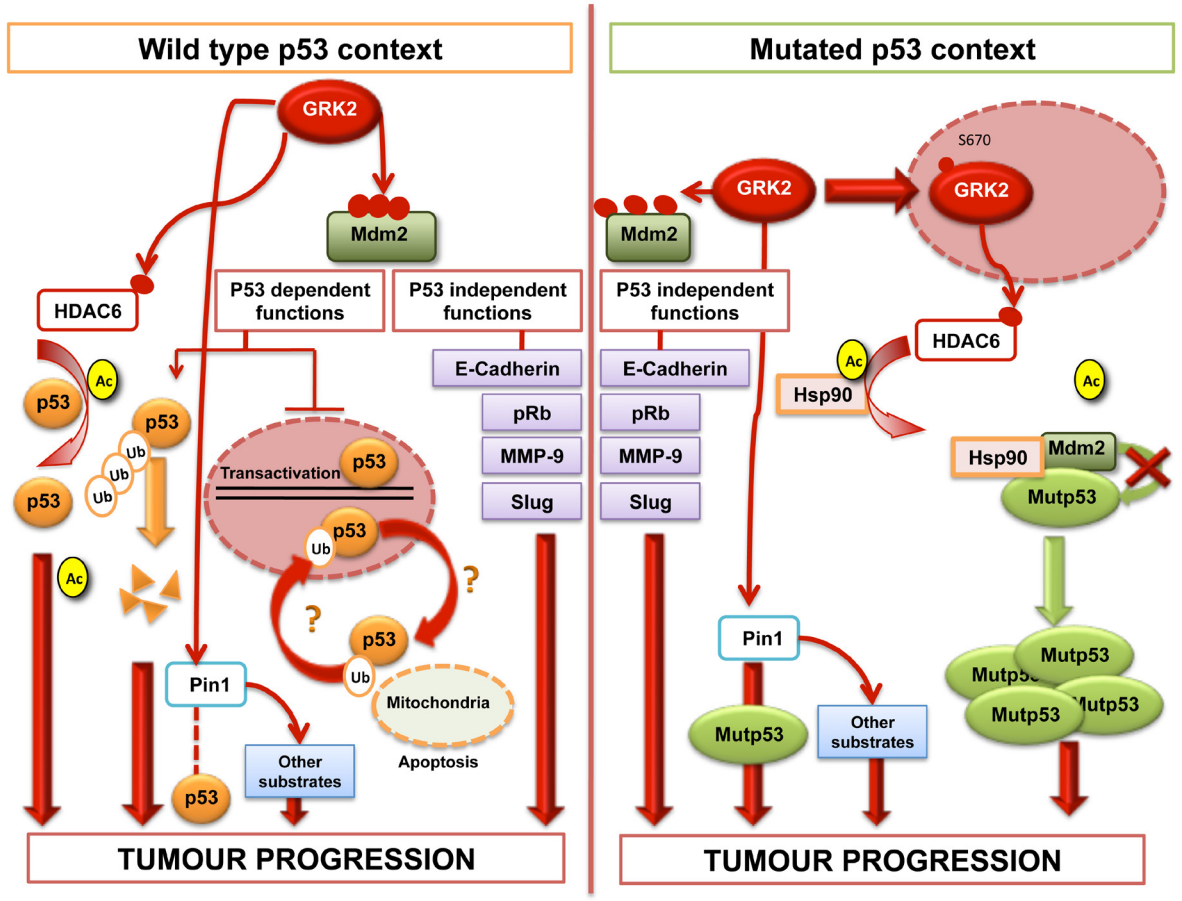


Figure D.4. Proposed model of GRK2 influence in tumour progression through the control of wild-type or mutated p53. In wild type p53 tumours, GRK2 would act as a negative modulator of p53 response through the phosphorylation and activation of Mdm2, main E3 ligase responsible of p53 degradation, and of HDAC6, which would promote the de-acetylation of p53, usually associated with tumour progression. On the other hand, GRK2 would contribute to mutant p53 stabilization through the regulation of HDAC6-Hsp90 axis and Pin1 expression. GRK2 would also potentiate p53-independent functions of Mdm2 in both wild-type and mutated p53 conditions.

Consistent with a role of GRK2/HDAC6 interaction in the control of mutant p53, we have demonstrated that different breast cancer cells harboring mutations of p53 accumulate higher levels of nuclear GRK2, which was phosphorylated at S670. This modification is not only instrumental for HDAC6 regulation and activation, but could be also relevant in the nuclear shuttling of the kinase, thus facilitating the formation of local specific GRK2 interactions for mutant p53 stabilization. However, the effect of GRK2 on the regulation of different mutants of p53 might be different according to the specific mutations and the subsequent conformational changes of the protein. In this sense, p53280R-K mutant (expressed in MDA-MB-231 cells) has been shown to be strongly dependent on Hsp90 regulation, whereas p53273R-H, a mutant p53 isoform present in MDA-MB468 cells, display lower ability to bind Hsp90 (Peng et al., 2001). Alternatively, those mutant p53

proteins intrinsically resistant to MDM2-mediated degradation could be also insensitive to GRK2. It has been reported that the interaction of Mdm2 with some mutant p53s differs from that of wild-type p53 as they bind to the RING-finger of the ligase through the DNA-binding domain instead of using their respective classical N-terminal domains (Lukashchuk & Vousden, 2007). This interaction prevents mutant p53 ubiquitination and degradation by Mdm2 since key structural determinants within the RING domain involved in p53 targeting to the proteaosome are blocked (Kulikov et al., 2010). Thus, stabilization of both Mdm2 and mutant p53 are not mutually exclusive (Cordon-cardo et al., 1994) and Mdm2 can provide pro-survival, proliferative and invasive properties in a p53-independent way (Manfredi, 2010).

In the search for additional players that might be involved in the GRK2 mediated modulation of the p53 response, it is worth noting the prolyl-isomerase Pin1. Notably, we find enhanced Pin1 expression upon GRK2 up-regulation in non-transformed cells and a positive correlation between both proteins in luminal breast cancer cells. Pin1 over-expression has been previously reported in human breast cancer tissues and its transcription is enhanced by oncogenic Neu or Ras (which signalling pathways are also potentiated by GRK2 expression) via E2F activation, otherwise stimulated by Mdm2 (Ryo et al., 2002; Wulf et al., 2001). Furthermore, Pin1 enhances the transformed phenotypes of Neu/Ras-transfected mammary epithelial cells (Ryo et al., 2002), and amplifies EGF signalling in breast cancer cells through its interaction with MEK1, leading to enhance HER-2 expression (Khanal et al., 2010). Moreover, Pin1 binds to different cell cycle and mitotic regulators, including GRK2 during G2/M transition (Penela et al., 2010b), acting as a central coordinator of cell cycle progression (Lu KP. 2000). The key functional feature of Pin1 is to tune different signalling cascades engaged in cellular proliferation and survival by selectively altering their signal duration and intensity. Such additional layer of regulation defines Pin1 as a relevant component of tumourigenic pathways. However, Pin1 also positively regulates numerous mediators of apoptosis. This means that Pin1 potentiates signal transduction irrespective of the particular cellular context, thereby reinforcing the alteration of cellular networks but not triggering them. For instance, Pin1 is required for full activity of wild-type p53, allowing its stabilization by preventing Mdm2 binding in response to DNA damage as well as by its ability to transactivate p21 and trigger cell cycle arrest an apoptosis (Penela et al., 2010b; Wulf et al., 2002). On the other hand, Pin1 can also stabilize p53 mutants with the same efficacy as the wild-type protein and promote the gain-of-function of mutant p53 by causing a profound qualitative change in its transcriptional program (Girardini et al., 2011). While in normal cellular settings Mdm2-mediated degradation of GRK2 and

Mdm2-dependent stabilization of p53 are coordinated by Pin1 in parallel, thus reinforcing the efficacy of cellular checkpoints, our data suggest that in luminal breast tumour cell lines the over-expression of GRK2 and Pin1 would tilt the balance between proliferative signalling and cell cycle checkpoint control towards uncontrolled proliferation irrespective of p53 status. Such co-up-regulation of GRK2 and Pin1 also points out that tumoural signalling switch the normal outcome of GRK2-Pin1 interaction (that is degradation) by mechanisms to be determined, but probably related to different modifications in Mdm2 (by AKT or Cdk2) or in Pin1 itself. Similarly, oncogenic signaling pathways override Pin1-mediated p53 stabilization in tumour cells that retain wild-type protein. In contrast, cells harbouring mutant p53 are addict to such functional aspect of Pin1, and in this context the cooperation between GRK2 and Pin1 might be more relevant. However, further studies are required to define the physiological and pathological roles of Pin1/GRK2-mediated p53 regulation.

4.

GRK2 is a central signalling node in cancer biology

In this work, we demonstrate that GRK2 plays a driver function in the development of central oncogenic features in breast cancer. As such, GRK2 should be considered as a transducing hub shared by different pathways actively sending and receiving signals. It has been noted that cancer-associated genes often constitute this type of nodes and are frequently involved in both positive and negative regulatory loops, which alteration drives prolonged activation of downstream effectors (for instance ERK or transcription factors) and the transition from a normal cellular sate into a long-term deregulated tumoural-prone state. Most of these cancer genes are interconnected and three hot areas have emerged in the cancer signalling network around RAS, p53 and TGFβ-signalling-dependent events (Cui et al., 2007). Interestingly, in such cancer-signalling map the more mutated genes a particular protein links to, the more probability of being a relevant cancer-associated onco-modulator one. GRK2 not only has numerous functional links to cancer-mutated genes but also interconnects and potentially modulate the hot areas mentioned above by means of the regulation of Mdm2 and HDAC6, thereby allowing the alteration of proliferation, survival and motility.

4.1. GRK2 levels and activity modulate breast tumour proliferation through HDAC6 and Mdm2-dependent mechanisms.

We show that increased GRK2 levels in untransformed MCF10A or 184B5 breast cells lead to an enhanced Ras loading and stimulation of ERK1/2 and Akt pathways in response to relevant growth factors such as heregulin or EGF, and to increased expression of Pin1 and phospho-His3 proliferation markers (both Ras-downstream effectors) in a way similar to the Ras/Neu induced transformation of breast cells. The over-activation of growth factor receptor signalling by extra GRK2 results in sustained proliferation. Moreover, up-regulation of GRK2 in transformed MCF7 orMDA-MB-231 cells enhances cell proliferation, markedly favours anchorage-independent growth and increases tumour growth in xenograft models in vivo. Moreover, this pro-mitogenic action of GRK2 requires its kinase activity, since the over-expression of a catalytically inactive mutant is not able to mimic such effects. Conversely, decreasing GRK2 levels hampers proliferation and anchorage-independent growth. Moreover, GRK2 down-regulation in MCF7 or MDA-MB-468 cells strikingly abrogates their ability to form tumours in vivo, indicative of a critical role for GRK2 in this process.

Notably, HDAC6 has been shown to be required for oncogenic Ras- and HER2-dependent fibroblast transformation (Lee et al., 2008) and for anchorage-independent growth of different cancer cells, including MCF7 (Lee et al., 2008; Mak et al., 2012). Moreover, HDAC6 knockout mice display reduced phosphorylation of ERK1/2 and Akt and lower levels of activated Ras (Aldana-Masangkay and Sakamoto, 2011). The influence of HDAC6 in these processes and signaling activities relies on deacetylation of several proteins (receptors, switchers, structural proteins, molecular chaperones, etc). It has been shown that enhanced HDAC6 activity reduces acetylation of Ras, an event required for efficient nucleotide exchange and for Ras-associated transforming activity (Yang et al., 2013). On the other hand, tubulin deacetylation contributes to the maintenance of EGFR at the plasma membrane by inhibiting MT-dependent receptor endocytosis, thus promoting sustained activation of downstream cascades and enhanced cell proliferation (Deribe et al., 2009; Gao et al., 2010). Our results point out GRK2 might affect the "mitogenic activity" of HDAC6 substrates by potentiating their deacetylation state in a HDAC6 phosphorylation-dependent manner. We show herein that an HDAC6 mutant unable to be phosphorylated by GRK2 (Lafarga et al., 2012a) inhibits the reinforcement of EGFR signalling to ERK1/2 observed in the presence of GRK2 up-regulation and leads to higher acetylated tubulin levels upon EGF challenge. Since both HDAC6 and GRK2 have been reported to interact with the EGFR (Chen et al., 2008; Deribe et al., 2009; Gao et al., 2005) we hypothesize that EGFR stimulation would promote a functional interaction

between GRK2 and HDAC6, leading to enhanced local deacytylase turnover, and more sustained EGFR downstream signalling (Fig.D.5). Such effect would be favoured by the higher GRK2 and HDAC6 levels present in luminal breast cancer cells (Fig.D.2). Notably, although less well characterized, it seems likely that the functional interaction between HDAC6 and GRK2 could be facilitated by relevant receptors other than the EGFR, since HDAC6 also interacts with other ErbB family receptors (Deribe et al., 2009), and with ER alpha estrogen receptors at or near the plasma membrane (Azuma et al., 2009), and ER stimulation can cause HDAC6-mediated tubulin deacetylation (Azuma et al., 2009), as well as GRK2-S670 phosphorylation (Dominguez et al., 2009).

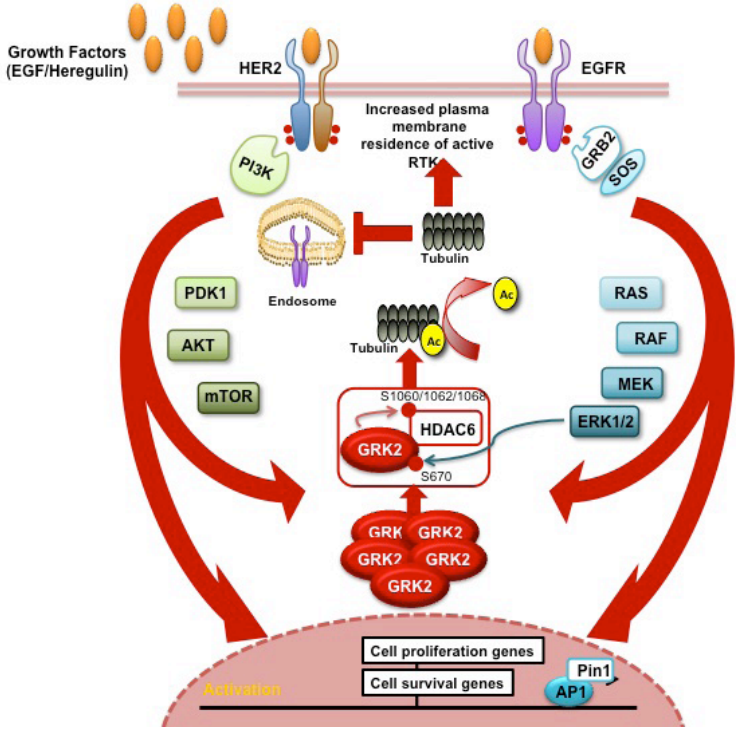


Figure D.5. Possible mechanism by which GRK2 enhances mitogenic pathways through the regulation of HDAC6.
Oncogenic up-regulation of the GRK2-HDAC6 interface favours the phosphorylation of HDAC6 by GRK2, which enhaces the deacetylase activity of HDAC6 towards tubulin. Enhanced activity of HDAC6 would block the EGFR (and probably other RTKs) internalization and degradation, resulting in increased plasma membrane residence of active RTKs, able to promote maintained mitogenic and survival signalling pathways.

On the other hand, novel functional aspects of Mdm2 have been described in relation to cellular proliferation and growth that involve the regulation of non-p53 key proteins such as Akt, E2F1, etc. in a ligase activity-independent manner. Thus, upregulation of Mdm2 promotes AKT phosphorylation irrespective of p53 status by repressing transcription of the PI3K regulatory subunit p85 (Singh et al., 2013). Down-modulation of p85 relies on the interaction of Mdm2 with the transcriptional activator REST and prevention of its loading on the p85 promoter. Interestingly, the central region of Mdm2 (aa 121-275) is required for AKT phosphorylation and several sites of GRK2-mediated phosphorylation have been identified in this region. In addition, the complex interplay between Mdm2 and E2F1 may allow GRK2 regulation of Mdm2 controlled S-phase entry and proliferation

in cancer cells. The N-terminal domain of Mdm2 contacts with E2F1 and stimulates its transcriptional ability to modulate genes required for S-phase progression (Gu et al., 2003; Martin et al.,1995). Mdm2 can also activate E2F1 indirectly by promoting the ubiquitination and degradation of RB (retinoblastoma protein), an E2F1 inhibitor (Xiao et al., 1995), while E2F1 stability is also enhanced by the Mdm2-mediated displacement of the ligase complex SCF-Skp2 from the transcriptional factor (Zhang et al., 2005). By increasing Mdm2 stability, GRK2 expression levels could positively influence these novel functional outcomes of Mdm2. Interestingly, a similar up-regulation of Mdm2 caused by the T309G polymorphism in the Mdm2 promoter has been correlated with tumour susceptibility and worse prognosis in some tumours, particularly in breast cancer (Bond et al., 2004). On top of that, Mdm2 is found to be one of the transcriptional targets of Ras/MAPK/ERK (Manfredi, 2010). Since GRK2 potentiates Ras activation in a kinase dependent manner, we hypothesize that a catalytically active GRK2 would be necessary to mediate Ras-dependent Mdm2 transcription, whereas the kinase independent impact of GRK2 on Mdm2 turnover could be controlled either by a scaffolding role of GRK2 or indirectly by the modulation of other proteins involved in the proteaosomal degradation of Mdm2. In sum, GRK2 is engaged in several positive feedback loops with HDAC6 and Mdm2, leading to activation of Ras and its downstream components AKT, ERK and PIN1, which are responsible for continuous activation of mitogenic pathways.

4.2. GRK2 confers resistance to diverse cellular insults and influences therapeutic responses in vitro.

In response to cellular insults, post-translational modifications occurring on MDM2 directly inhibit its E3 ligase function or restrain this protein in certain sub-cellular locations such as the nucleolus to relieve p53 from degradation (Bernardi et al., 2004; Shi & Gu, 2012). As a consequence, p53 is strongly accumulated, increasing cell cycle arrest and apoptotic responses. We put forward that the regulatory role of GRK2 on Mdm2 and HDAC6-dependent tumoural competences would endow cells with an improved ability to resist p53-mediated cell death induced by different stresses (Hanahan & Weinberg, 2011). First, by decreasing p53 stability and activation, increased GRK2 expression in non-transformed cells enhances growth potential under nutrient starvation stress as well as resistance to cell death induced by common chemotherapeutic agents such as etoposide, cisplatin, paclitaxel or doxorubicin. Conversely, down-regulation of GRK2 sensitizes transformed breast cancer cells (MCF7) to these apoptotic stimuli. Second, extra levels of GRK2 in different breast cancer cells with different molecular signatures lessen both proliferation arrest and cytotoxic effectiveness of either Nutlin3-a (in MCF7 cells: wt p53, high levels of GRK2)

or HDAC6 inhibitors such as tubacin (in non transformed 184B5 cells: wt p53, normal levels of GRK2) or SAHA (in both 184B5 and MDA-MB-231 cells: mutant p53, normal levels of GRK2). It is tempting to speculate that phosphorylation of Mdm2 by GRK2 could potentiate Mdm2/p53 interaction or promote allosteric alterations in the ligase that reduce affinity or conceal the binding site for Nutlin3a. In addition, modification of the central region and CAD domain by GRK2 could also affect the interaction of Mdm2 with other pro-arresting and pro-apoptotic proteins, either positively such as p21, Nsb1, Rb, p300 (which are targeted for degradation or inhibition), or negatively, such as ARF , thus resulting in different cellular responses that converge in fostering cell survival. Parallel to such GRK2-mediated p53-dependent and -independent effects of Mdm2 in survival, phosphorylation of HDAC6 by GRK2 could also strength p53 deacetylation, what would promote the attenuation of p53 transcriptional activity and its degradation (Ding et al., 2013; Ozaki et al., 2013), thereby resulting in increased cancer cell survival (Namdar et al., 2010). Interestingly, HDAC inhibitors markedly increase the acetylation state of p53, which is essential for p53-mediated cell growth arrest and apoptosis (Tang et al., 2008).

Therefore, it is tempting to suggest that GRK2-mediated phosphorylation and activation of either Mdm2 or HDAC6 makes these molecules less sensitive to their inhibitors, thereby debilitating their therapeutic function. Such notion would be consistent with ineffective or partial responses shown by Nutlin3-a or by pan-HDAC6 inhibitors in GRK2-overexpressing ER-positive luminal cells (MCF7, MDA-MB361) (Tate et al., 2012,). Moreover, pan-HDAC inhibitors also display different efficacies among breast tumour cells with p53 mutant status. Notably, panobinostat (Novartis) elicits effective responses in triple-negative breast tumour cells (MDA-MB231, MDA-MB157), which do not display enhanced GRK2 levels but not in cells with high kinase levels such as MDA-MB468 (Tate et al., 2012). Finally, it must be taken into account that the positive action of GRK2 in the regulation of Mdm2-p53 axis may not synergize with its role as enhancer of HDAC6 activity in some tumoural situations, such as those involving mutant p53 tumours. In this context, stable over-expression of MDM2 enhances SAHA- induced degradation of mutant p53 whereas the specific MDM2 inhibitor Nutlin-3a partially prevents SAHA-induced destabilization of mutp53 (Li et al., 2011b). These evidences suggest that GRK2 expression might be a relevant factor in the heterogeneous response to chemotherapeutic drugs noted in tumour cells with variable p53 status, Mdm2 functionality or HDAC6 content. Thus, a better understanding of the functional relationships between these proteins will help to delineate future therapeutic strategies of interest aimed to either manipulate the catalytic activity of GRK2 or the GRK2 -Mdm2/GRK2-HDAC6 interfaces. Notably, besides the Mmd2 and HDAC6 connections, the ability of GRK2 to interact with other molecules

such as Akt, p38, Smad2/3 or RKIP would also contribute to the anti-apoptotic effects observed in transformed breast cancer cells upon treatment with chemotherapeutic agents.

Overall, our observations may have profound implications from a therapeutic perspective, as the extent of GRK2 expression could modify the clinical outcome of breast cancer treatments in patients. To date, strategies to induce p53 activation in tumours that retain wild-type p53, which represent 80% of all breast cancers, are promising cancer therapies. However, for the remaining 20% of tumours with mutant p53, which are mainly classified as triple-negative cases, there are not molecular-targeted therapeutic strategies so far. Moreover, this group of tumours is particularly refractory to chemotherapy and at risk to progress to metastatic breast cancer. On top of that, even in the more treatable groups of breast cancer patients the success of therapies is limited by the development of drug resistances. In fact, although ER-positive tumours are suitable for endocrine therapies, most develop resistance to treatment, which is also common in Her2-targeting therapies. Multiple lines of evidence suggest that these phenomena can be explained by the presence of complex bidirectional cross-talks between the ER and HER2pathways (Nahta & O'Regan, 2012; Tokuda et al., 2012). Thus, treatment strategies targeting either pathway are associated with up-regulation of the other one. In addition, antagonists of the "classic" nuclear ER (tamoxifen, raloxifene and ICI182,780), are also agonists of GPR30, what may induce unexpected proliferative responses. As a result, the efficacy of the tamoxifen therapy on luminal estrogen receptor-positive breast tumours is hampered in one-third of the women treated by mechanisms depending on increased growth factor receptors and PI3K signaling. Since GRK2 is emerging as a central onco-modulator of key tumoural processes such as cell proliferation and survival through its role in the control of global p53 responsiveness or its strong connection with a wide variety of oncogenic pathways, it is feasible that GRK2 could be involved in the development of these resistances. Therefore, we can hypothesize that breast cancer treatments would benefit from the combination of well known molecular-targeted treatments and chemotherapies with the inhibition of GRK2 or specific GRK2-mediated molecular interactions.

4.3. GRK2 promotes cancer cell invasion and stromal remodelling

Also relevant to our understanding of cancer progression is the ability of tumour cells to migrate and disseminate to distant organs, a main cause of death in breast cancer patients. Interestingly, while GRK2 is up-regulated in circa 40% of patients of a general cohort with infiltrating ductal carcinoma, this figure raises up to 80% in patients that have experienced metastasis to lymph nodes. A similar trend is noted in Mdm2, which is up-regulated more frequently in metastatic tumours than in primary ones (Datta et al., 2001). Therefore, both GRK2 and Mdm2 might play a relevant role in tumour cell migration

and invasion. In support of this, over-expression of GRK2 in the highly metastatic MDA-MB-231 cells or in non-transformed breast cancer cells, potentiates either Heregulin or EGF-induced chemotaxis. However, GRK2 down-modulation in MDA-MB-231 cells does not affect cell migration in a collagen type I surface, suggesting that the overall impact of GRK2 on cell migration is not straightforward and varies depending on the stimuli considered. This is consistent with previous findings showing that GRK2 promotes cell motility towards fibronectin in fibroblast or epithelial cells, whereas migration of these cells towards collagen type IV was unaltered (Penela et al., 2008). The final outcome of GRK2 in cell migration would depend on how its activity is engaged and integrated into signalling pathways that control different steps of the chemotactic process (receptor sensing, cell polarization, membrane protrusion, adhesion/de-adhesion cycles) in a cell type-specific and stimuli-dependent context, as both positive and negative effects or no effect of GRK2 in motility have been reported (Penela et al., 2014a). GRK2 could facilitate leading edge formation and cellular displacement of polarized mammary epithelial cells in a multifaceted way. First, it might amplify the intensity and duration of pro-migratory signalling downstream chemotactic receptors (EGF or S1P receptors) and integrins; second, GRK2 can controls microtubule dynamics through the activation of HDAC6; third, it promotes reorganization of the actin cytoskeleton by stimulating ERM proteins and fourth, GRK2 increases focal adhesion turnover via induction of GIT1-scaffolding functions (Fig.D.6A) (Penela et al., 2014 and references therein).Consistently, extra levels of GRK2 potentiate GIT-1 expression levels and activation of MAPK and Ras in mammary 184B5 cells as well as HDAC6 activity and tubulin deacetylation, all together contributing to build up a robust chemotactic response.

Striking features of the movement of mammary metastatic cells in vivo are their high speed during locomotion on ECM fibers (due to weak adhesion contacts) and their high degree of persistent lineal motion (related to a higher polarization ability) compared to the random migration of less-efficient metastatic cells (Condeelis & Segall, 2003). Therefore, reduced expression of GRK2 could lessen the efficacy of metastatic movement in vivo by limiting polarization and favouring tight adhesion. Consistently, our results indicate that upon GRK2 down-regulation random walking of MDA-MB-231 cells is increased (perhaps indicative of a desensitizing role of GRK2 on some autocrine- activated receptors in the absence of guiding cues), while cell migration towards Heregulin or CCL21 is impaired (Fig.D.6B). Interestingly, expression of extra GRK2 levels in non-transformed 184B5 breast cells strongly decreases E-cadherin expression levels, while those of vimentin are clearly up-regulated. These findings suggest the occurrence of an epithelial-mesenchymal transition-like process that could rely on GRK2 interplay with known factors leading to EMT, such as Mdm2 or components of the TGFb1 transducing pathway. Mdm2 promotes

cell migration and invasion through the ubiquitination and degradation of E-Cadherin (Yang et al., 2006), by neutralizing the MDM2 binding protein (MTBP)-mediated suppression of tumourigenesis and metastasis, as well as by positively regulating the mRNA of Slug, VEGF and MMP9 proteins (Chen et al., 2013b; Jung et al., 2013b).

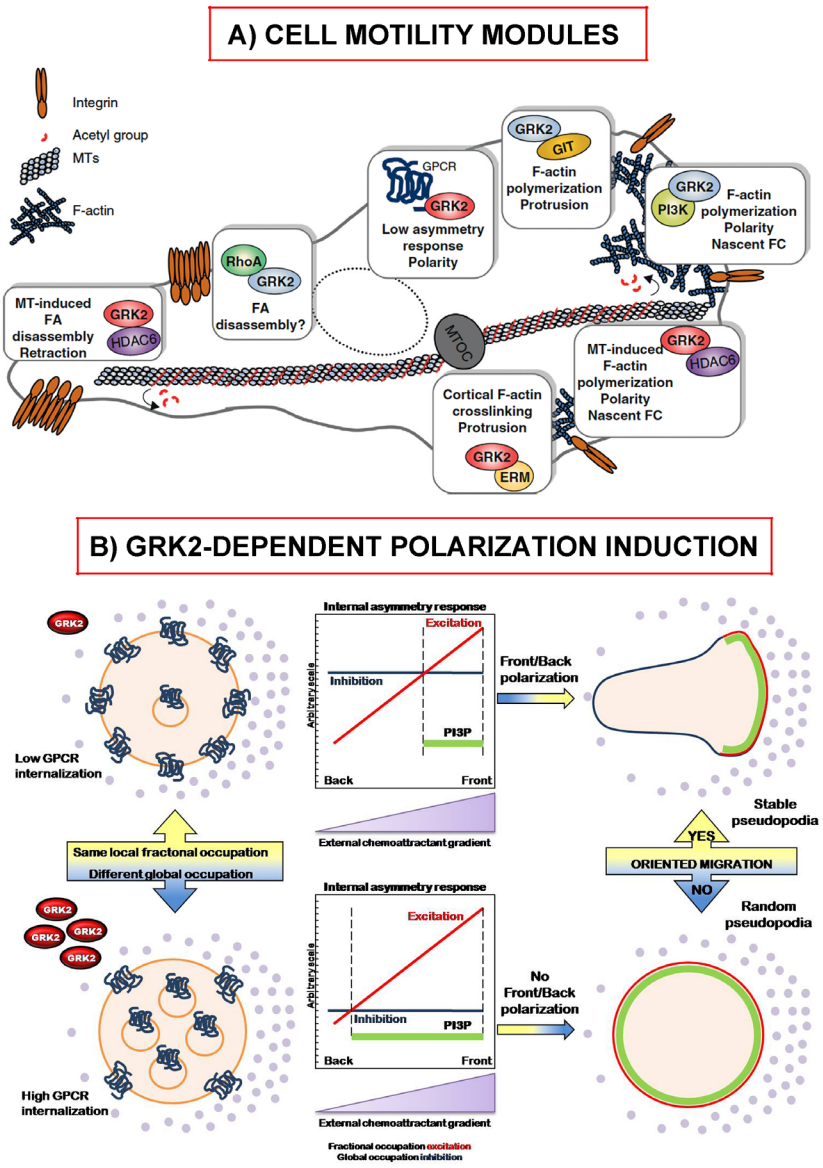
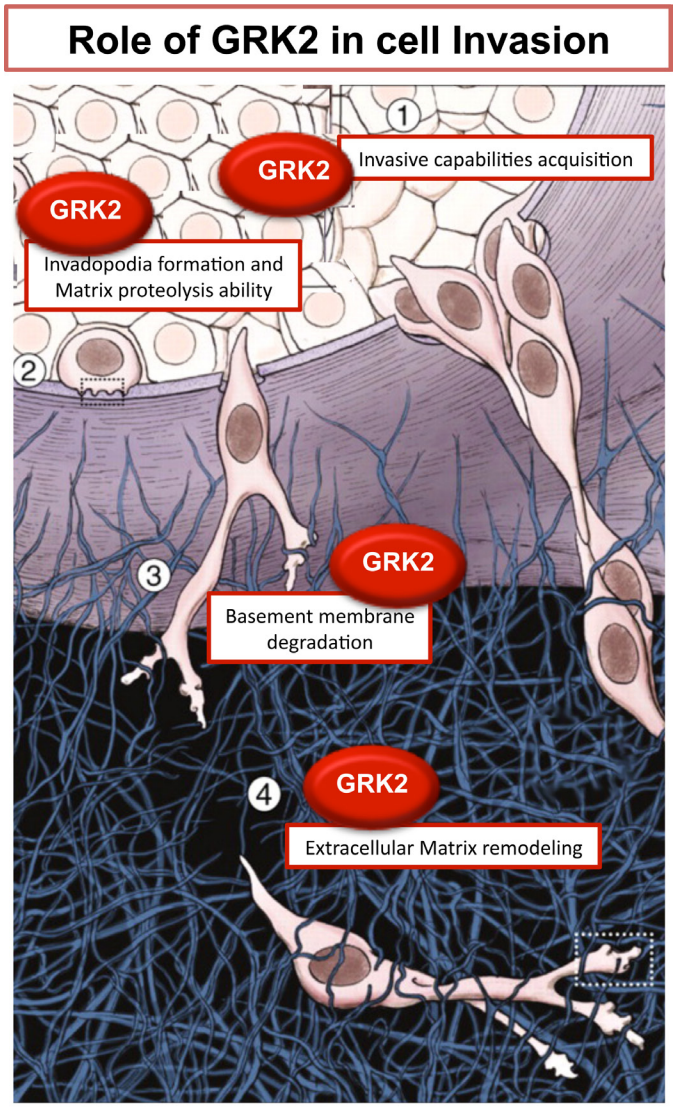


Figure D.6. Role of GRK2 in cell migration. A) By dynamically interacting with diverse substrates and effectors in different cellular locations, GRK2 may modulate several facets of the cell migration machinery in a stimuli and cellular context-dependent manner. FA, focal adhesions; FC, focal contacts. B) Proposed model for the role of GRK2 in the induction of cell polarization. Local fraction of receptor occupancy by chemoattractants would trigger stimulatory signals for the generation of internal signalling asymmetry, whereas global levels of occupied receptors would inhibit this parameter. In the presence of lower GRK2 levels/activity, stimulated GPCR would remain longer/more functional in the membrane, thus eliciting robust directional sensing responses and the specification of stable pseudopodia as if the gradient was steeper. Conversely, excessive GRK2 activity would diminish the global level of occupied receptors, resulting in the inability to differentiate the cell front from the back, as occurs in the absence of gradients, resulting in cessation of movement.

During in vivo-monitored invasion into the surrounding tissue (Condeelis & Segall, 2003), mammary tumour cells traverse long distance walking on ECM fibers that result from both tumoural- and stromal- (fibroblasts, immune cells) dependent microenvironment remodelling and by following EGF gradients. Cells often converge in the proximity of vessels, also endowed with a basement membrane and a dense coating of ECM that must be degraded or "squeezed" for intravasation. We have demonstrated that GRK2 is necessary for the breakdown of both basement membrane and extracellular matrix (ECM), since silencing of GRK2 in MDA-MB-231 cells hampers cell invasion to matrigel (similar to basement membrane) and to collagen type I (mimicking ECM) in 2D and 3D models of invasion. Moreover, GRK2 abrogation blocks the capability of tumoural cells to degrade gelatin, a process that it strictly dependent on MT1-MMP metaloproteinase activity and on formation of protrusive structures called invadopodia (Artym et al., 2006b) (Fig.D.7). This result suggests a role for GRK2 in the control of MT1-MMP expression or trafficking and/ or in the structural formation/maturation of invadopodia themselves. Underpinning these processes might be the functional interplay of GRK2 with p53, Mdm2 and/or HDAC6. It has been described that functional p53 inhibits the invasive capacity of tumoural cells by repressing several metaloproteinases such as MMP1, MMP2 or MMP13 (Ala-aho et al., 2002; Kim et al., 2010b), whereas mutated p53 has the opposite effect (Adorno et al., 2009), suggesting that GRK2-dependent modulation of the p53/Mdm2 axis could contribute to cell invasion. The involvement of this regulatory axis in these cellular processes is strengthened by the anti-invasive effect of inhibitors of the of Mdm2-p53 interface as nutlin-3 (Polanski et al., 2010). Interestingly, on the basis that (mono)-ubiquitination is a key modification in protein internalization and vesicular trafficking, that Mdm2 mediates mono-ubiquitination of several substrates and mediates the internalization of membrane proteins (GPCRs or E-Cadherin), and the novel finding showing that ubiquitination of MT1-MMP impacts on metalloproteinase trafficking (Eisenach et al., 2012), it is tempting to suggest a role for Mdm2/ GRK2 by means of the ubiquitination of MT1-MMP (Eisenach et al., 2012), or of other proteins related with its intracellular trafficking.

Besides its potential role in the endowment of invadopodia with proteolytic activity, GRK2 might be also required for their structural assembly by means of the modulation of HDAC6 and/or GIT-1. HDAC6 is a key player of invadopodia formation through the regulation of tubulin and cortactin. Deacetylation of cortactin is essential for generation of a dendritic actin network to form the core of the nascent and elongating invadopodia, while microtubule dynamics linked to tubulin deacetylation is needed for MT1-MMP re-routing and exocytosis (Castro-Castro et al., 2012; Rey et al., 2011). The growth of invadopodia and their extension deeper into the substratum is permitted by the infiltration

of microtubules, which in combination with an intact vimentin filament network support invadopodia elongation and stabilization (Schoumacher M et al., 2010 JBC). Interestingly, dynamic microtubules appear to be restricted to the base of invadopodia, while in the stem and apex they are stable (Schoumacher et al., 2010). It is feasible that GRK2-mediated regulation of HDAC6 contributes to gradient stability of invadopodial MTs. Moreover, up-regulation of vimentin caused by GRK2 could also favor invadopodia formation in mammary cells. Akin to vimentin, GIT-1 is up-regulated in cells with extra GRK2. GIT has been reported to assemble lipid and protein kinases and small GTPase signaling complexes in podosomes (invadopodia-related structures) upon Src activation, an event necessary and sufficient for the induction of invadopodia and podosome formation. In sum, we speculate that the positive effects of GRK2 in invasive motility of breast cancer cells could be mediated by HDAC6, GIT1 or Mdm2 activities.



Adapted from Poincloux R et al. J Cell Sci 2009

Figure D.7. GRK2 contributes to different sequential steps of the metastatic cascade. First, GRK2 potentiates the adquisition of invasive capabilites in tumoural cells. Then, GRK2 favours invadopodia formation and matrix proteolytic activity to breach the basement membrane, followed by ECM remodeling, in which the kinase has also a positive role.

Initial escape of metastatic cells from the tumour mass rely on an intrinsic cell-autonomous altered behaviour, which is conditioned by extrinsic microenvironmental factors such as the interaction of malignant cells with the extracellular matrix (ECM) and with cellular components of the tumoural stroma that release signals responsible for fostering chemotaxis and invasiveness. Our group has recently reported that GRK2 levels might affect tumour progression by altering such interplay between malignant cells and their surrounding microenvironment. Transformed breast epithelial cells (with high levels of GRK2) can trigger in a paracrine way down-modulation of GRK2 specifically in the surrounding tumour endothelium, what results in vasculature dysfunction and more aggressive tumour progression (Rivas et al., 2013). Tumour-associated vessels with lower GRK2 content display deficient pericyte-endothelial attachment and increased permeability, blood leakage and tortuosity, leading to inefficient tissue oxygenation and nutrient supply. Notably, the increased hypoxic tumoural microenvironment induces infiltration of immune cells such as macrophages, which also contribute to increase angiogenesis by releasing endothelial activating factors. These features would escalate extravasation of immune cells, which positively contribute to tumour growth, but also intravasation of tumour cells. Moreover, the interplay between GRK2 and Mdm2 could be also relevant in angiogenesis, since up-regulation of Mdm2 in epithelial transformed cells stimulates the production and secretion of pro-angiogenic factors such as VEGF (Narasimhan et al., 2008; Inada et al., 1996). Therefore, concurrent and opposite changes of GRK2 in the epithelial (up-regulation) and stromal (down-modulation) components of breast tumours might act synergistically to develop tumour-promoting inflammation and genome instability, two characteristics that enable cancer cells the acquisition of hallmarks of cancer, and that finally results in tumour survival, growth, and dissemination (Hanahan & Weinberg, 2011). A better knowledge of the mechanisms underlying such cell type–specific modulation and roles of GRK2 may help to understand its integrated role in cancer development and to design novel therapeutic strategies.

5.

Future directions

Due to the overall integrative effect of GRK2 in the acquisition of tumoural features and its participation in the majority of hallmarks of cancer (Fig.D.8), we hypothesize a possible role of this kinase in the emerging attributes of cancer cells important for cancer development such as **reprogramming energy metabolism.** In this context, GRK2 is associated with insulin resistance and obesity (Garcia-guerra et al., 2010), both risk factors in breast tumourigenesis, especially in post-menopausal women (Thomson et al., 2009). The metabolic context of obesity and diabetes as a risk factor of breast cancer involves the inhibition of p53 response. Moreover, p53 directly influence various metabolic pathways, including AMPK and mTOR signalling, carbohydrate and lipid metabolism, the regulation of autophagy, and the maintenance of mitochondrial integrity and REDOX balance enabling cells to respond to metabolic stress and counteracting many of the metabolic alterations associated with cancer development (Berkers et al., 2013). Interestingly, GRK2 diminishes glucose uptake and blocks insulin signalling through the phosphorylation and kidnapping of the insulin receptor substrate protein IRS1. Since Mdm2 mediates IRS1 degradation (Usui et al., 2004), we speculate a possible role of GRK2 in the Mdm2-mediated IRS1 degradation. However, GRK2 could

also impact on cell metabolism through the control of Mdm2-mediated regulation of p53. Overall, all these evidences put forward that the role of GRK2 in cancer progression could be more complex than expected and deserves a detailed characterization in each particular scenario.

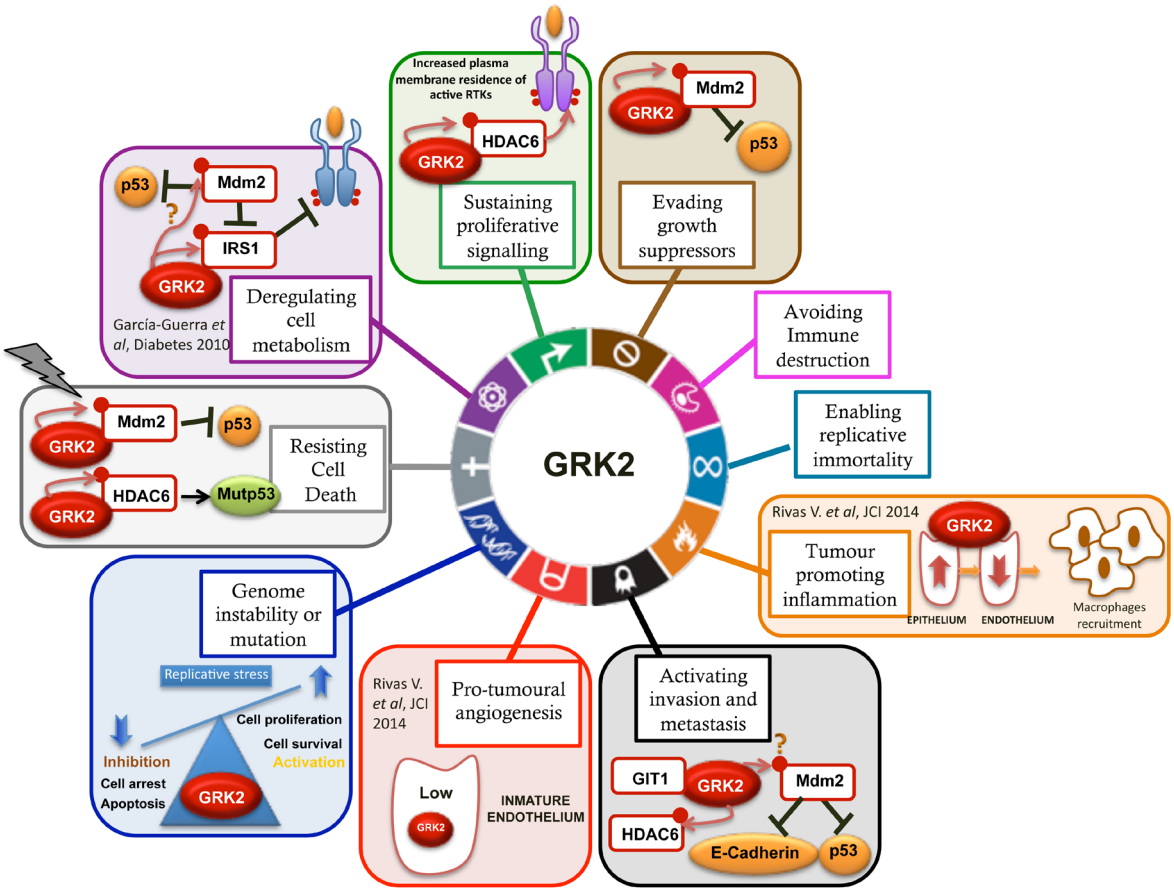


Figure D.8. GRK2 favours the adquisition of hallmarks of cancer. Schematic representation of the role of GRK2 as a main contributor to the development of cancer.

CONCLUSIONS

1. The overexpression of GRK2, HDAC6 and Mdm2 emerges as a common functional module of luminal breast cancer.

2. The main oncogenic pathways that trigger luminal breast tumour transformation increase GRK2 levels in these cancer types.

3. GRK2 is a new Mdm2-activating kinase. GRK2 phosphorylates Mdm2 at several sites, stabilizes the Mdm2 protein, promotes its nuclear localization and enhances the binding between p53 and the ligase, thus regulating the p53 response in a negative way.

4. GRK2 favours the acquisition of breast cancer hallmarks by enhancing cell proliferation and survival, by decreasing the p53-mediated apoptotic responses and by promoting the invasive migration of breast tumoural cells. These effects of GRK2 involve the modulation of HDAC6 and/or Mdm2-dependent processes.

5. GRK2 is a key mediator of "in vivo" tumour growth promoted by either wild-type or mutated p53 transformed breast cancer cells.

CONCLUSIONES EN CASTELLANO

1. La sobreexpresión de GRK2, HDAC6 y Mdm2 surge como un módulo funcional común en el cáncer de mama de tipo luminal.

2. Las principales vías de señalización oncogénicas responsables de la transformación tumoral de mama tipo luminal tales como receptores tirosina quinasa, el eje PI3K/AKT o receptores de estrógenos confluyen en el aumento de los niveles de GRK2 en este tipo de tumores.

3. Mdm2 es un nuevo sustrato de GRK2. GRK2 fosforila a Mdm2 en múltiples residuos, aumenta la estabilidad de la ligasa, promueve su localización nuclear y facilita la unión Mdm2-p53, regulando de forma negativa la respuesta de p53.

4. GRK2 favorece la adquisición de capacidades tumorales mediante el aumento de la proliferación celular y la supervivencia, la disminución de las respuestas apoptóticas mediadas por p53 y la promoción de la migración invasiva de las células tumorales de mama. Entre los mecanismos moleculares responsables del efecto de GRK2 en la progresión tumoral se encuentran la modulación de HDAC6 o la regulación del eje p53/Mdm2.

5. GRK2 es un mediador clave del crecimiento tumoral "in vivo", independientemente del estatus de p53.

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ABBREVIATIONS

αARs	alfa adrenergic receptors
β-arr	β-arrestin
β1AR	beta-1 adrenergic receptor
β2AR	beta-3 adrenergic receptor
aa	Amino acid
ADH	Atypical ductal hyperplasia
ADP	Adenosine diphosphate
AKT	protein kinase B
ATP	Adenosine triphosphate
AP1	Activator protein 1
BM	Basal membrane
BRCA	Breast Cancer Type 2 susceptibility protein
BSA:	Bovine serum albumin
CAD	Central acidic domain
CCL21	Chemokine (C-C motif) ligand 21
CDK2	Cyclin-dependent kinase 2
CHX	Cicloheximide
CK1	Casein kinase 1
CXCR	C-X-C chemokine receptor type
DAB	Diaminobenzidine
DAG	Diacylglycerol

DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracelular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
Elite ABC kit	Avidin-biotinperoxidase complex
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine-Nucleotide Exchange Factor
GF	Growth factor
GIT	G-protein-coupled receptor kinase-interacting protein
GPCR	G-Protein coupled receptor
GRB2	Growth factor receptor-bound protein 2

GRK	G-protein coupled kinase
GSK3	Glycogen synthase kinase 3
GPR30 **GPER1**	G protein-coupled receptor for estrógeno 30 G protein-coupled estrogen receptor 1
HDAC6	Histone deacetylase 6
HER2	Human Epidermal growth factor Receptor-type2
HIF	Hypoxia-inducible factors
HS	Horse serum
HSP90	Heat shock protein 90
IGFR	Insulin-like growth factor 1 receptor
IDC	Infiltrating ductal carcinoma
IP	Immunoprecipitation
IP3	Inositol trisphosphate
Iso	Isoproterenol
LC/ MS	Liquid chromatography–mass spectrometry
MAPK	Mitogen-activated protein kinases
MDM2	Murine double minute 2
MMP	Matrix metallopeptidase
MT1-MMP	Membrane type 1-matrix metalloproteinase 1
mTOR	Mammalian target of rapamycin
NaVO3	Sodium orthovanadate
NES	Nuclear export signal

NLS	Nuclear localization sequence
PDK1 **PDPK1**	Phosphoinositide-dependent kinase-1 3-phosphoinositide-dependent protein kinase 1
PCR	Polymerase chain reaction
PH domain	Pleckstrin homology domain
Pi	Inorganic phosphate
PI3K	Phosphoinositide 3-kinase
PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PIP2	Phosphoinositol biphosphate
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase
PVDF	Polyvinylidene fluoride
RGS	Regulators of G protein signaling
RING	Really Interesting New Gene
RNA	Ribonucleic acid
S1P	Sphingosine-1-phosphate
SDF1a	Stromal cell-derived factor α
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SH domain	Src homology domain
shRNA	A small hairpin RNA or short hairpin RNA

siRNA	Small interfering RNA
SOS	Son of sevenless
S1P	Sphingosine-1-phosphate
TAE	Tris base, acetic acid and EDTA
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Thr	Threonine
TKR	Tyrosine kinase receptors
Tyr	Tyrosine
Ub	Ubiquitin
WB	Western blot
WT	Wild-type